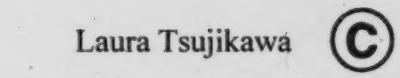
UNIVERSITY OF ALBERTA

THE EFFECT OF AN ETHYL PHOSPHOTRIESTER ON DNA REPLICATION *IN VITRO*

BY

Laura Tsujikawa 

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Abstract

Alkylating agents cause DNA damage that reduces the fidelity of DNA replication. The exogenous alkylating agent, ethylnitrosourea (ENU), predominantly ethylates the phosphate backbone of DNA resulting in the production of ethyl phosphotriesters. To improve our understanding of the effect of an ethyl phosphotriester on DNA replication, oligonucleotides containing a single ethyl phosphotriester were used as templates for an *in vitro* primer extension assay catalyzed by wild type and mutant T4 DNA polymerases and *Escherichia coli* DNA polymerase I. DNA replication by wild type T4 DNA polymerase was severely hindered by the presence of an ethyl phosphotriester, with limiting nucleotide concentration. A further comparison of the T4 DNA polymerase mutants, D112A/E114A exonuclease deficient and I417V polymerase defective DNA polymerases, suggests that an ethyl phosphotriester slows elongation and promotes 3'→5' exonuclease activity. DNA replication by *Escherichia coli* DNA polymerase I, however, was not hindered at the site of the ethyl phosphotriester.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Effect of an Ethyl Phosphotriester on DNA Replication *In Vitro* submitted by Laura Tsujikawa in partial fulfillment of the requirements for the degree of Master of Science in

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List of Symbols

α	alpha
γ	gamma
μ	micro

List of Abbreviations

A	adenine
ATP	adenosine triphosphate
A	alanine
D	aspartate
Cys	cysteine
C	cytosine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNMP	deoxynucleoside monophosphate
dNTP	deoxynucleoside triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
ENU	ethylnitrosourea
E	glutamate
G	guanine
HPLC	high pressure liquid chromatography
PPi	inorganic pyrophosphate
I	isoleucine
Ipr-Pac	isopropylphenoxyacetyl
L	leucine
M	methionine
MNU	methylnitrosourea
µl	microlitre
µM	micromolar
mM	millimolar
nM	nanomolar
NMR	nuclear magnetic resonance
O^6-mG	O^6-methylguanine
O^6-MGMT	O^6-methylguanine DNA methyltransferase
Pac	phenoxyacetyl
RNA	ribonucleic acid
SV40	simian virus 40
SCE	sister chromatid exchange
SN_1	substitution nucleophilic reaction type 1
TLC	thin layer chromatography
T	thymine
Tris-HCl	tris hydroxymethyl aminomethane hydrochloride
UV	ultraviolet
V	valine
W	watt

Introduction

The propagation of all species depends upon the inheritance of genetic information. Prior to its transmission from parent to offspring, genetic information must first be copied. DNA polymerases are the enzymes responsible for the replication of DNA, by catalyzing the template-directed incorporation of deoxyribonucleotides. The incorporation of nucleotides occurs in a 5'→ 3' direction where a phosphodiester bond is formed between the 3'-OH group of the primer terminus and the 5'-α phosphate of the incoming deoxyribonucleoside triphosphate (dNTP) (Kornberg *et al.*, 1956). This results in the addition of a deoxyribonucleoside monophosphate (dNMP) and the release of pyrophosphate (PPi) (Figure 1). DNA polymerase-catalyzed DNA replication is highly accurate, exhibiting an error frequency of only 10^{-5}-10^{-6}. In addition to accurate nucleotide incorporation, an exonucleolytic proofreading mechanism is associated with most DNA polymerases, which further reduces the error frequency to 10^{-8} (Schaaper, 1993). Exonuclease activity occurs in a 3'→ 5' direction and is responsible for removing nucleotides that have been misincorporated (Figure 1). Whether or not proofreading will predominate over nucleotide incorporation, depends upon the status of the primer-terminus. If elongation is slowed by the presence of a mismatch or DNA damage at the primer-terminus, then exonucleolytic proofreading will prevail. On the other hand, if the primer-terminus is free of damage or mismatched base pairs then nucleotide incorporation predominates.

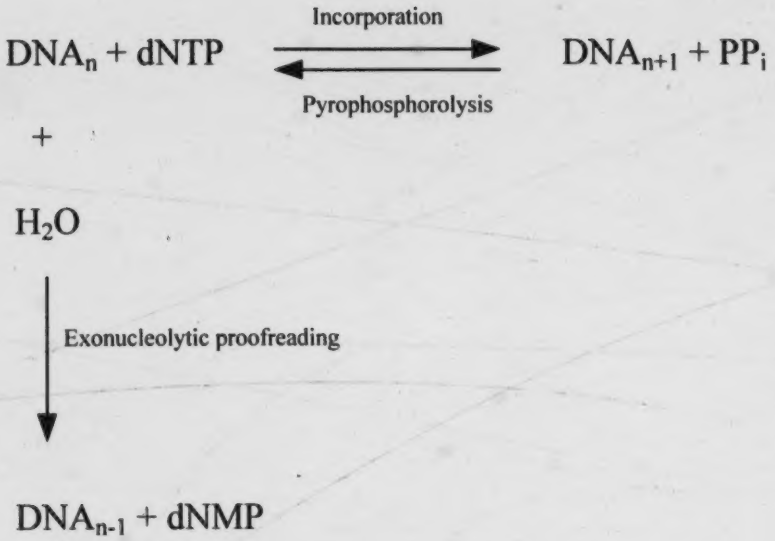


Figure 1: **Reactions catalyzed by DNA polymerases during DNA replication.** DNA polymerase-catalyzed DNA replication consists of 5'→3' nucleotide incorporation, pyrophosphorolysis, and 3'→5' exonucleolytic proofreading. The primer DNA containing n number of nucleotides (DNA_n) is extended by the incorporation of a nucleotide dictated by the template. The DNA polymerase catalyzes the formation of a phosphodiester bond between the 3'-OH group of the primer terminus and the incoming dNTP (Kornberg *et al.*, 1956). A dNMP is added extending the primer by 1 (DNA_{n+1}) and PP_i is released. The reverse reaction of nucleotide incorporation is pyrophosphorolysis. Exonucleolytic proofreading occurs when an incorporated dNMP is removed from the primer, reducing it by 1 (DNA_{n-1}). This reaction requires H_2O.

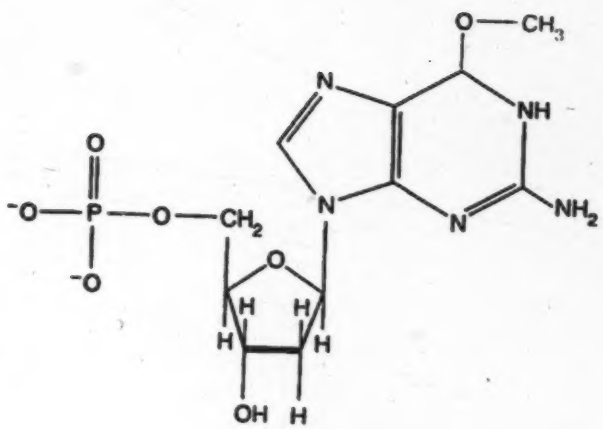
Both accurate nucleotide incorporation and exonucleolytic proofreading are necessary for high fidelity DNA replication. With the additional safety of post-replicative repair pathways, the number of errors that occur in the DNA are sufficiently low to sustain both survival and evolution of a species.

There are, however, many endogenous and exogenous DNA damaging agents that affect the fidelity of DNA replication. These agents can produce adducts on the DNA that are mutagenic, carcinogenic, and even cytotoxic. Natural metabolic processes such as lipid peroxidation produce some of these endogenous alkylating agents (Reviewed by Vaca *et al.*, 1988).

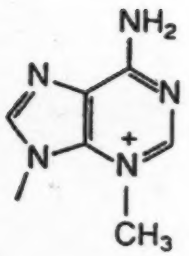
A variety of adducts are produced in DNA after exposure to an alkylating agent. The most abundant lesions formed, however, are 7-alkylguanine, O^6-alkylguanine, 3-alkyladenine, and alkylphosphotriesters (Figures 2 and 3). Due to their asymmetrical nature, phosphotriesters can exist in the Rp or Sp configuration (Figure 3).

Both prokaryotic and eukaryotic cells expend enormous amounts of energy to repair DNA damage caused by endogenous alkylating agents. In *Escherichia coli* an inducible DNA repair pathway, known as the adaptive response, enables cells to display increased resistance to the deleterious effects of alkylating agents (Samson and Cairns, 1977; Teo *et al.*, 1986). The key enzyme in this inducible repair pathway is the product of the *ada* gene, O^6-methylguanine DNA methyltransferase (O^6-MGMT) (Demple *et al.*, 1982). This suicide enzyme irreversibly transfers the methyl group from O^6-methylguanine (O^6-mG) to a C-

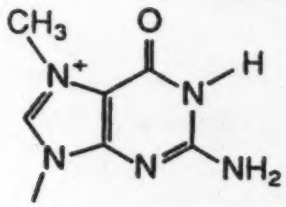
Figure 2: Structures of O^6-methylguanine, N_3-methyladenine and N_7-methylguanine.



O^6-methylguanine



N_3-methyladenine



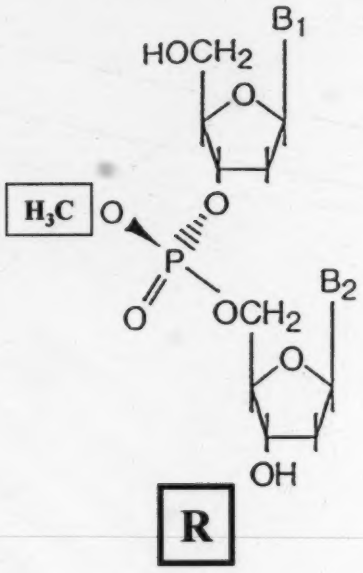
N_7-methylguanine

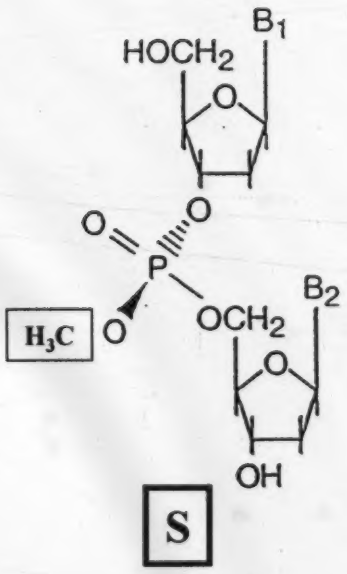
terminal cysteine residue, Cys-321, thus rendering itself inactive (Demple *et al.*, 1985). In addition to repairing O^6-mG, O^6-MGMT also transfers a methyl or ethyl group from an alkylated internucleotide phosphate in the Sp configuration to an N-terminal cysteine residue, Cys-69 (Hamblin and Potter, 1985; Weinfeld *et al.*, 1985; Teo *et al.*, 1986).

The mechanism by which the Ada protein only recognizes or repairs the Sp isomer of alkylphosphotriesters is unknown. It has been suggested that the Sp isomer is more accessible to the Ada protein since this adduct would protrude from the backbone of DNA, whereas the Rp isomer is imbedded in the major groove in closer proximity to the bases (Kan *et al.*, 1973; Pramanik and Kan, 1987; Weinfeld *et al.*, 1985). Also, when DNA is treated with ENU *in vitro* more of the Sp isomer of alkylphosphotriesters is generated than the Rp isomer (Jensen and Reed, 1978).

The second role of the Ada protein begins when the enzyme accepts a methyl group from the Sp isomer of a methyl phosphotriester and is converted into a strong transcriptional activator of the genes induced in the adaptive response; *alkA*, *alkB*, *aidB*, and of its own gene, *ada* (Nakabeppu and Sekiguchi, 1985; Teo *et al.*, 1986). The *alkA* gene product is a 3-methyladenine DNA glycosylase, which repairs 3-methyladenine, along with 3-methylguanine, O^2-methylcytosine, and O^2-methylthymine (McCarthy *et al.*, 1984). Unrepaired, 3-methyladenine blocks both DNA and RNA polymerases (Larson *et al.*, 1985), which leads to sister chromatid exchange (SCE) and cell death (Engelward *et al.*, 1998). The *alkB* gene product is also involved in the excision repair of alkylated bases

Figure 3: Structures of the Rp and Sp isomers of a methyl phosphotriester.





(Kataoka and Sekiguchi, 1985), while the biochemical function of the *aidB* gene product remains unknown (Volkert and Nguyen, 1984).

In order to transcriptionally activate all of the genes required in the adaptive response, the methylation of Cys-69 of the O^6MGMT, through the repair of S-methylphosphotriesters must occur (Teo *et al.*, 1986). It is interesting that the repair of what is thought to be an innocuous lesion is absolutely required for the activation of the adaptive response and subsequent survival of the organism (McCarthy *et al.*, 1983; Margison *et al.*, 1985). The adaptive response of *E. coli* is also active against DNA damage caused by ethylating agents (Sedgewick and Lindahl, 1982), many of which produce predominantly phosphotriesters.

Alkyl phosphotriesters are among the most abundant lesions formed after exposure to many ethylating agents (Engelse *et al.*, 1986; Engelse *et al.*, 1987). For example, ethyl phosphotriesters comprise 56-70% of the total lesions produced after exposure to ethylnitrosourea (ENU) *in vitro* (Sun and Singer, 1973; Jensen and Reed, 1978) and *in vivo* (Bodell *et al.*, 1979). ENU is characterized as an SN_1 alkylating agent, which means that it has a tendency to alkylate the oxygen atoms rather than the nitrogen atoms of DNA (Table 1) (Veleminsky *et al.*, 1970).

As well as being abundant, alkyl phosphotriesters, and in particular ethyl phosphotriesters, are known to be chemically stable at physiological pH and appear not to be repaired in mammalian cells. Ethyl phosphotriesters can be detected in human cells 72 hours (2-3 cell doublings) after initial exposure to ENU (Bodell *et al.*, 1979).

Table 1: Alkylation products produced by a variety of alkylating agents

Compound	Percent of total alkylation[a]		
	N_7-guanine	O^6-guanine	Phosphotriesters
Dimethylsulphate	79	---	0.1-1
Diethylsulphate	62-67	0.2-1	0-16
Methylmethanesulfonate	82	0.3	0.3-0.82
Ethylmethanesulfonate	58	2	12-14
Isopropylmethanesulfonate	7.6	2.5	90
Methylnitrosourea	66	5.5	12-14
Ethylnitrosourea	11-14	8.1	56-70
Dimethlnitrosamine	68	6	11

[a] Data obtained from Weinfeld, M. (1982).

In addition, ethyl phosphotriesters are the most abundant lesion remaining in most tissues of male Wistar rats 56 days after exposure to ENU (Engelse *et al.*, 1987), while the half life for this persistent adduct in the liver of ENU-treated adult rats is 32 days (Engelse *et al.*, 1986).

When cells are treated with alkylating agents, the RNA also becomes alkylated. However, phosphotriesters in DNA are far more stable than in RNA, where their production leads to chain scission and subsequent loss of infectivity in bacteriophage R17 and Tobacco Mosaic Virus (Shooter, 1975; Singer *et al.*, 1975).

The persistence of alkyl phosphotriesters in DNA is further exemplified by their resistance to a wide variety of exo- and endonucleases, such as, DNase I, DNase II, micrococcal nuclease, S1 nuclease, snake venom exonuclease, calf spleen phosphodiesterase (Jensen and Reed, 1978; Miller *et al.*, 1971), and restriction endonucleases (Gallo *et al.*, 1986). In addition, the presence of ethyl phosphotriesters in the operator regions of DNA significantly reduces the ability of repressor protein binding (Siebenlist *et al.*, 1980; Takeda *et al.*, 1983), slows RNA synthesis *in vivo* (Marushige and Marushige, 1983), and completely abolishes RNA polymerase binding to promoter sequences *in vitro* (Siebenlist and Gilbert, 1980; Jorgensen *et al.*, 1991). Ethyl phosphotriesters also inhibit the action of T4 polynucleotide kinase (Weinfeld and Livingston, 1986) and are refractory to the binding of *Thermus aquaticus* mutS (Biswas and Hsieh, 1997), human AP endonuclease (Wilson *et al.*, 1997), HeLa nuclear factor I (Vries *et al.*, 1987), Herpes simplex virus type 1 origin-binding protein (Simonsson *et al.*, 1998), SV40

large T antigen (Jones and Tjian, 1984), XerD recombinase (Blakely *et al.*, 1997), and mutY (Lu *et al.*, 1995).

In addition, ethyl phosphotriesters have been implicated in the induction of SCEs (Morris *et al.*, 1983; Heflich *et al.*, 1982; Tates *et al.*, 1983). SCE has been shown to occur only when DNA damage is present during S phase (Wolff *et al.*, 1974). To elicit SCE, the DNA replication apparatus may be stalled or blocked, leading to chromosome gaps and breaks (Bender *et al.*, 1973). There is just one study in the literature that addresses the effect of phosphotriesters on DNA replication *in vitro*. Miller *et al.* (1982) reported that DNA replication by *E. coli* DNA polymerase I is slowed by the presence of an ethyl phosphotriester and that the Rp isomer affected DNA replication more than the Sp isomer. The finding that the Rp is more inhibitory than the Sp isomer is not surprising since the former is in closer proximity to the bases directly involved in nucleotide incorporation (Kan *et al.*, 1973; Pramanik and Kan, 1987; Weinfeld *et al.*, 1985).

Ethyl phosphotriesters are chemically stable adducts, resistant to a wide variety of exo- and endonucleases, inhibit the binding of many DNA-binding proteins, significantly slow DNA replication by *E. coli* DNA polymerase I, and appear to escape repair in mammalian cells. Therefore, it is likely that phosphotriesters within the DNA backbone are not simply innocuous adducts and further study is warranted to determine their role in both DNA replication and cellular responses to alkylating agents.

My thesis involved studying the effects of an ethyl phosphotriester on replication by DNA polymerases, including wild type and mutant T4 DNA

polymerases and *E. coli* DNA polymerase I. My hypothesis was that an ethyl phosphotriester would have a more pronounced affect on DNA replication by T4 DNA polymerase, which replicates the chromosome, than on DNA replication by *E. coli* DNA polymerase I, which is a repair polymerase. An *in vitro* primer extension assay was used since the *in vivo* effects of ethyl phosphotriesters on DNA replication are difficult to determine because alkylating agents produce a variety of adducts at a variety of positions in the DNA (Singer, 1976; Reviewed by Lawley, 1995).

Bacteriophage T4 DNA polymerase has been extensively used in DNA replication studies because it has been well characterized biochemically (Capson *et al.*, 1992; Bloom *et al.*, 1994; Frey *et al.*, 1995) and genetically (reviewed by Reha-Krantz, 1995). In addition, the crystal structure has been solved for the RB69 DNA polymerase, which is 63% identical to T4 DNA polymerase (Wang *et al.*, 1997). Through a series of elegant classical genetic experiments, mutator and antimutator DNA polymerases were identified (Speyer *et al.*, 1966; Drake *et al.*, 1969). Mutator DNA polymerases (Speyer *et al.*, 1966), which often have reduced 3'→5' exonuclease activity (Muzyczka *et al.*, 1972; Reha-Krantz and Nonay, 1993) replicate DNA with less accuracy than the wild type enzyme. Mutations that decrease the polymerase's ability to prepare the primer terminus for exonucleolytic proofreading activity also produce a mutator phenotype (Baker and Reha-Krantz, 1998; Stocki *et al.*, 1995; Marquez and Reha-Krantz, 1996). Antimutator DNA polymerases (Drake and Allen, 1968; Drake *et al.*, 1969), which

proofread more frequently, replicate DNA with greater accuracy than the wild type enzyme (Muzyczka *et al.*, 1972; Gillin and Nossal, 1976).

Having too little or too much exonucleolytic proofreading is not advantageous to an organism. Mutator DNA polymerases produce a high level of mutations due to reduced exonucleolytic proofreading activity (Reha-Krantz *et al.*, 1986; Reha-Krantz, 1988). On the other hand, DNA polymerases with the antimutator phenotype waste nucleotides and replicate DNA more slowly than wild type DNA polymerases (Clayton *et al.*, 1979). Thus, the fidelity of DNA replication relies heavily on a balance between nucleotide incorporation and exonucleolytic proofreading (Muzyczka *et al.*, 1972; Clayton *et al.*, 1979).

Local DNA structure can also affect the fidelity of DNA replication (Pless and Bessman, 1983; Bloom *et al.*, 1993). Any adduct on the DNA that slows extension may elicit the transfer of the DNA from the polymerase active centre to the exonuclease active centre. In this case, DNA polymerases may have difficulty bypassing the lesion but those DNA polymerases that are proficient in exonuclease activity may also be forced to "idle". Idling occurs when a DNA polymerase repeatedly incorporates a nucleotide and then excises it (Hershfield and Nossal, 1972). This non-productive cycle results in the depletion of nucleotide pools and slower extension (Reviewed by Reha-Krantz, 1998). Wild type T4 DNA polymerase has a potent 3'→5' exonuclease activity and is, therefore, predicted to "idle" at the site of the ethyl phosphotriester. Antimutator T4 DNA polymerases should have the greatest difficulty bypassing an ethyl phosphotriester since they proofread more often than wild type T4 DNA polymerase, whereas mutator T4

DNA polymerases should have the least difficulty because they proofread less often.

Two mutant T4 DNA polymerases, D112A/E114A and I417V, were tested to determine if a mutator or an antimutator enzyme, respectively, could replicate DNA containing an ethyl phosphotriester. The mutator DNA polymerase, D112A/E114A, contains alanine substitutions at highly conserved amino acid residues in the exonuclease active centre. Aspartate-112 is required to coordinate metal ions in the exonuclease active centre. Therefore, this mutant can attempt to proofread but it is defective in performing the hydrolysis reaction and exhibits a residual exonuclease activity that is 1000-fold less than wild type T4 DNA polymerase (Reha-Krantz and Nonay, 1993). The antimutator DNA polymerase, I417V, contains a valine substituted for an isoleucine in the highly conserved motif A region (Delarue *et al.*, 1990), which forms part of the polymerase active centre. The I417V amino acid substitution reduces DNA binding in the polymerase active centre and promotes the binding of DNA in the exonuclease active centre (Reviewed by Reha-Krantz, 1998; Reha-Krantz and Nonay, 1994).

Unlike the highly processive wild type T4 DNA polymerase (Reddy *et al.*, 1992), polymerase to exonuclease active site switching by *E. coli* DNA polymerase I requires the enzyme to dissociate and then rebind the primer terminus for the most part (Joyce, 1989). In addition, *E. coli* DNA polymerase I has both 5'→3' and 3'→5' exonuclease activities (Kornberg, 1980), although the

3'→5' exonucleolytic proofreading activity is 1000-fold less active than that of wild type T4 DNA polymerase (Capson *et al.*, 1992).

The use of mutant DNA polymerases to study the effect of an ethyl phosphotriester on DNA replication can be used to further probe DNA polymerase function and to gain insight into what role ethyl phosphotriesters play in the global response of an organism to alkylating agents. With our *in vitro* primer extension assay, template locations that cause the DNA polymerase to pause or stop completely can be observed. Determining if the DNA polymerase "senses" the ethyl phosphotriester, resulting in slowed elongation and subsequent initiation of proofreading, will provide insight into DNA replication in general.

Materials and Methods

Synthesis, deprotection, and purification of oligonucleotides

The synthetic oligonucleotides used in this study are described in Table 2. Dr. M. Weinfeld provided the phosphotriester-containing DNAs, PT1, PT2R and PT2S, and the unmodified control DNA, C2. The synthesis of the decamer oligonucleotide bearing the phosphotriester (PT1) and the identical control sequence lacking a phosphotriester has been described previously (Weinfeld and Livingston, 1986). Briefly, the dinucleotide Tp(Et)T was prepared by cesium fluoride catalyzed transesterification of the 2-chlorophenylphosphotriester of 5'-protected TpT (Chattopadhyaya and Reese, 1980). The ethyl phosphotriester was then incorporated into a fully protected decamer by solution-phase synthesis (Chattopadhyaya and Reese, 1980), using the bis(triazolide) of 2-chlorophenyl phosphate as the phosphorylating agent and 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole as the condensing reagent. Deprotection of the oligonucleotides was effected by sequential treatment with tetramethylguanidinium-4-nitrobenzaldoximate to remove 2-chlorophenyl groups, ammonia to remove the benzoyl protecting groups from deoxyadenosine and deoxycytidine, and acetic acid to remove the 5'-methoxytrityl protecting group. The decamers were purified by HPLC on an anion exchange column, and characterized by NMR and two-dimensional mobility shift analysis (Jay *et al.*, 1974)

The 32-mer oligonucleotides containing a single site-specifically ethylated phosphate (PT2R and PT2S) were kindly provided by Dr. Elena Atrazheva (Cross Cancer Institute). The first step in the synthesis was the preparation of the 5'-

protected phosphate-ethylated dinucleotide Gp(Et)T by cesium fluoride catalyzed transesterification as described above for Tp(Et)T. The Rp and Sp stereoisomers of protected ethylated dinucleotide were separated by chromatography on silica gel. These were reacted with 2-cyanoethyl N,N,N'N'-tetraisopropyl phosphoramidite to generate the 3'-2-cyanoethyl-diisopropylphosphoramidite of the 5'-protected isomers of Gp(Et)T, which then served as reagents in the synthesis of the protected 32-mers by standard phosphoramidite chemistry on an oligonucleotide synthesizer at the DNA synthesis laboratory at the University of Calgary. The base protecting groups used throughout the synthesis, including the preparation of the dinucleotide, were iso-propylphenoxyacetyl (iPr-PAC) for guanine, phenoxyacetyl (PAC) for adenine, and isobutyryl for cytosine. These groups were removed at the same time as releasing the oligonucleotide from the solid-phase support by incubating the oligonucleotide-bearing beads in 25% aqueous ammonia for 20 minutes at 60 °C. (When it was discovered that, due to a side reaction during oligonucleotide synthesis, the iPr-PAC groups had been partially substituted by acetyl groups, the deprotection was extended by 3-3.5 hours.) The oligonucleotides were purified by polyacrylamide gel electrophoresis (Sambrook *et al.*, 1989) and ultraviolet shadowing (see below). A control oligonucleotide lacking a phosphotriester (C2) was synthesized using the same PAC protecting groups and deprotected under the same conditions. The stereochemical configuration of the ethyl phosphotriester in each oligonucleotide was established by incubating the oligonucleotides, radiolabeled at the 5'-terminus, with *E. coli* O^6-MGMT and then digesting the oligonucleotide with the 3'→5'

exonuclease of T4 DNA polymerase. The digestion products were examined by gel electrophoresis. (The methyltransferase has the capacity to remove methyl and ethyl groups from the DNA phosphate backbone, but only when in the Sp configuration (Weinfeld *et al.*, 1985). The removal of the alkyl group allows the exonuclease to complete digestion of the oligonucleotide to the terminal dinucleotide. If the Rp phosphotriester is still present, exonuclease digestion stalls at, or close to, the site of the phosphotriester.)

All other oligonucleotides were synthesized, using standard solid-phase phosphoramidite chemistry by the DNA Synthesis Lab in the Department of Biological Sciences. These oligonucleotides were purified by gel electrophoresis on a polyacrylamide gel, containing 15% acrylamide, 7 M urea and TBE buffer (100 mM Tris [pH 8.3], 100 mM boric acid, and 2 mM EDTA). Typically, 50 μg of DNA was loaded on a 1.5 mm thick gel and run at 45 W for 80 minutes. Ultraviolet shadowing, using TLC silica plates (F_{254} sheets, EM industries), was performed to visualize the DNA bands. Bands corresponding to full-length oligonucleotides were excised from the gel and transferred to Eppendorf tubes. The gel slices were crushed in 200-500 μl of buffer containing 0.5 M sodium-acetate, 100 mM Tris [pH 8.0], and 5 mM EDTA. The DNA was eluted by shaking at 37 °C overnight. The tubes were centrifuged for 30 seconds to pellet the gel pieces. The DNA-containing supernatant was run through a C-18 Sep-pak cartridge (Waters, Millipore). First the column was charged by the addition of 20 ml of 100% methanol and equilibrated with 10 ml of buffer (100 mM Tris [pH 8.0] and 5 mM EDTA). After the DNA was applied to the column by gravity, the

column was washed first with 10 ml of buffer (100 mM Tris [pH 8.0] and 5 mM EDTA) and then with 10 ml of Millipore water. The DNA was eluted from the column with 6 ml of 40% methanol; 1.5 ml fractions were collected. A speed-vacuum dryer was used to concentrate the DNA in each fraction. The DNA pellets were resuspended in 40-50 µl of TE buffer (10 mM Tris [pH 8.0] and 1 mM EDTA) and stored at –20 °C. Oligonucleotide concentration was determined by measuring absorbance at 260 nm. The extinction coefficient for each oligonucleotide was calculated from the sequence composition.

DNA polymerases and exonucleases

Purification and characterization of the wild-type bacteriophage T4 DNA polymerase, the mutant D112A/E114A-DNA polymerase (Reha-Krantz and Nonay, 1993), and the mutant I417V-DNA polymerase (Reha-Krantz and Nonay, 1994) have been described (Refer to Table 3). The DNA polymerases were diluted in buffer containing 50% glycerol, 0.1 M potassium phosphate [pH 6.9], 1 mM dithiothreitol, and 0.05 mM EDTA. *Escherichia coli* DNA polymerase I was purchased from Gibco-BRL and Exonuclease III was purchased from New England Biolabs.

Kinase reactions

Oligonucleotides at 0.3-2 pmol/µl were labelled at the 5' end by T4 polynucleotide kinase (Boehringer Mannheim) with 3 pmols of [γ^{32}P] ATP (3000 Ci/mmol, Dupont NEN) in the presence of kinase buffer (50 mM Tris [pH 7.6], 10 mM $MgCl_2$, 5 mM dithiolthreitol, 0.1 mM spermidine, 0.1 mM EDTA) (Maniatis

et al., 1982). The reaction mixes were incubated at 37 °C for 30 minutes and stopped by boiling for 2 minutes.

Annealing reactions

Kinased oligonucleotides were annealed to unlabelled oligonucleotides present in 1.5-fold excess in the presence of 50 mM NaCl and 25 mM Tris [pH 8.0]. The reaction mix was incubated at 70 °C for 5 minutes and then allowed to cool slowly to room temperature (approximately 2 hours).

Primer extension assay

Labelled primer-template substrate (Figure 4) at 1 nM was incubated with 5 or 100 μM dNTPs, 1 mM dithiothreitol, 70 mM Tris [pH 8.0], 18 mM $(NH_4)_2SO_4$, 200 μg/ml bovine serum albumin, and 7 mM $MgCl_2$. Reaction mixes were pre-incubated at 37° C for 1 minute and the reactions were started with 0.1-20 nM DNA polymerase. Reactions were stopped by adding an aliquot of the reaction mix to an equal volume of loading dye (40% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 1 mM NaOH, 1 mM EDTA) on ice. The reaction products were separated by electrophoresis on a 15% or 20% denaturing polyacrylamide gel run at 45 W for 80 minutes. The gels were dried and then applied to PhosphorImager screens (Molecular Dynamics) for analysis.

Single-stranded and double-stranded DNA exonuclease assays

Labelled single-stranded or double-stranded templates at 1-20 nM were used in the exonuclease assays. These experiments were performed under the same conditions as the primer extension assays with the omission of dNTPs. The

reactions were started with 0.1-10 nM wild-type T4 DNA polymerase or 10 nM Exonuclease III. Reactions were stopped by boiling for 2 minutes and then the reaction products were electrophoresed on a 15% or 20% denaturing polyacrylamide gel at 45 W for 70 minutes. The gels were dried and then applied for 5 hours to PhosphorImager screens (Molecular Dynamics).

Construction of the PT1* DNA substrate

The decamer, PT1, (25 μM) was ligated to Lrk-224 (50 μM) (Table 2), by first annealing both oligonucleotides to a complementary 25-mer bridge (250 μM) (Table 2) in the presence of 50 mM NaCl and 25 mM Tris [pH 8.0]. The reaction mix was incubated at 70 °C for 5 minutes and then allowed to cool slowly to room temperature. The entire annealing reaction was then incubated with 50 mM Tris [pH 7.6], 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 5% polyethylene glycol-8000, and 2 units of T4 DNA ligase (Gibco-BRL) at 4 °C for 3 days. The reaction was stopped by the addition of 5 μl of loading dye (40% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 1 mM NaOH) and boiling for 2 minutes. The ligation products were run on a 1.5 mm 12% polyacrylamide gel, containing 7 M urea and Tris-borate-EDTA buffer [pH 8.3] at 60 W for 4 hours. The bridge/marker (Lrk 225) is the same length as the ligated DNA but does not contain a 3'- phosphate. Thus, only the bridge was degraded with 1 μM wild-type T4 DNA polymerase in the presence of 8 mM $MgCl_2$, 200 μg/ml bovine serum albumin, and 25 mM Tris [pH 8.0] at 37 °C for 1 hour. Reactions were stopped by boiling for 5 minutes and the DNA was precipitated by the addition of 0.25vol of 3

M sodium acetate (pH 7.5) and an equal volume of 100% ethanol. DNA pellets were washed with 70% ethanol and resuspended in 10-40 µl of TE (10 mM Tris [pH 8.0], 1 mM EDTA). Ligated products were purified through a C-18 Sep-pak cartridge (Waters) as previously described.

Quantitation of reaction products by densitometry

A PhosphorImager and ImageQuant software (Molecular Dynamics) were employed to calculate band intensities. From these calculations, the percentages of full-length product, primer and significant intermediate products were determined.

Table 2: DNA Oligonucleotides Synthesized for the Primer Extension and Exonuclease Assays

Template Oligonucleotides

PT1 5' TTT ^{*a} TCTATTT 3'

PT1* 5' TTT * TCTATTTCCCGTCCACTTCGCCp 3'

PT2R 5' TAGTGATAAATTAAAG ^{R*} TCTTGCGCCTTAAACC 3'

PT2S 5' TAGTGATAAATTAAAG ^{S*} TCTTGCGCCTTAAACC 3'

Primer Oligonucleotides

P1 5' GGCGAAGTGGACGGG 3'

P2 5' GGTTTAAGGCG 3'

P3 5' GGTTTAAGGCGCAAGACTTT 3'

Unmodified Control Oligonucleotides

C 5' TTTTCTATTTCCCGTCCACTTCGCCp^{b} 3'

C1^{c} 5' TAGTGATAAATTAAAGTCTTGCGCCTTAAACC 3'

C2^{c} 5' TAGTGATAAATTAAAGTCTTGCGCCTTAAACC 3'

Complementary Oligonucleotide

Comp1^{d} 5' GGTTTAAGGCGCAAGACTTTAATTTATCACTAp^{b} 3'

Oligonucleotides used in construction of PT1*

Lrk-224 5' CCCGTCCACTTCGCCp^{b} 3'

Bridge 5' GGCGAAGTGGACGGGAAATAGAAAA 3'

[a] * = ethyl phosphotriester in either R or S conformation R*= R isomer S*= S isomer

[b] p = phosphate

[c] C1 and C2 are identical in sequence composition but were synthesized by different procedures (see Materials and Methods).

[d] Comp1 is complementary to C1, C2, PT2R, and PT2S

Table 3: Characterization of the DNA polymerases used in the primer extension and exonuclease assays.

DNA Polymerase	Characteristics	Relative Exonuclease activity
Bacteriophage T4		
Wild type	5'→3' polymerase and 3'→5' exo activity	1[a]
D112A/E114A	Low 3'→5' exo activity . Alanine substitutions for highly conserved amino acid residues. Aspartate-112 is a ligand for metal ions in the exo active centre. This mutant exhibits a mutator phenotype.	0.001
I417V	Potent 3'→5' exonuclease activity Valine is substituted for isoleucine in motif A which forms part of the pol active centre. This mutant has reduced binding of DNA in the pol active centre, resulting in increased binding of DNA in the exo active site, a high nucleotide turnover rate, low processivity, and an antimutator phenotype.	3[b]
Escherichia coli		
DNA pol I	5'→3' exonuclease activity Low 3'→5' exonuclease activity Less processive than wild type T4 DNA polymerase.	0.001

a = exonuclease activities are relative to the 3'→5' exonuclease activity of the wild type T4 DNA polymerase.

b = turnover rate relative to wild type T4 DNA polymerase under processive conditions (Reha-Krantz and Nonay, 1994).

Figure 4: Primer template substrates used in this study and in Miller's study.

A) Primer template substrates used in this study.

Part 1:

Primer P1 5' GGCGAAGTGGACGGG 3'
Template C **3'pCCGCTTCACCTGCCCTTTATCTTTT 5'**

Primer P1 5' GGCGAAGTGGACGGG 3'
Template PT1* **3'pCCGCTTCACCTGCCCTTTATCT*TTT 5'**

Part 2:

Primer P2 5' GGTTTAAGGCGCAAGA CTTT 3'
Templates C2, PT2R, and PT2S **3' CCAAATTCCGCGTTCT*GAAATTAAATAGTGAT 5'**

Primer P3 5' GGTTTAAGGCG 3'
Templates C2, PT2R, and PT2S **3' CCAAATTCCGCGTTCT*GAAATTAAATAGTGAT 5'**

* = Position of the ethyl phosphotriester.

B) Primer template substrates used in Miller's study.

Primer 5' $(pT)_8$ CC 3'
Template **3' $(pA)_{11}GGTTA^{Rp}GAACC$ 3'**

Primer 5' $(pT)_8$ CC 3'
Template **3' $(pA)_{12}GGTTA^{Sp}GAACC$ 3'**

Results

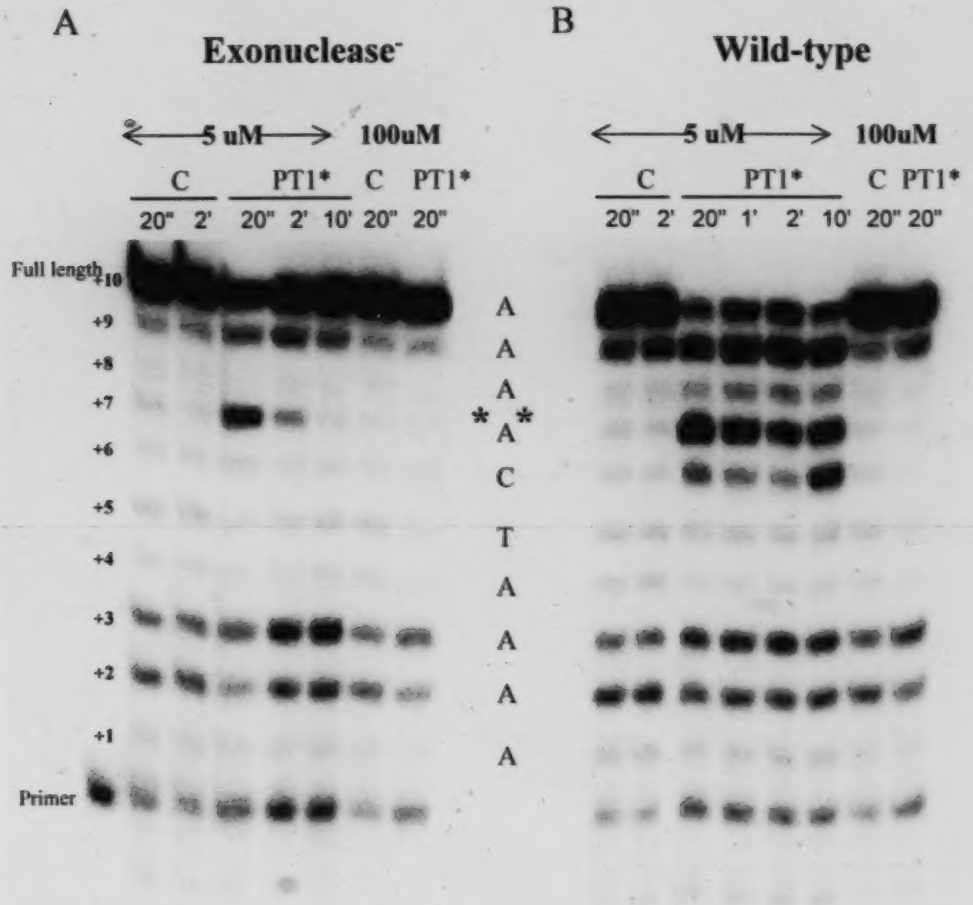
Part 1: The effect of an ethyl phosphotriester on DNA replication using PT1* as a template

A) *DNA replication by the exonuclease deficient D112A/E114A-T4 DNA polymerase is paused by an ethyl phosphotriester in the DNA template in the presence of low dNTP concentration.*

Initial assays were performed with the exonuclease deficient D112A/E114A-T4 DNA polymerase (Exo$^-$) (Table 3). This was done so that nucleotide incorporation could be focused on without the additional complexity of 3'→5' exonucleolytic degradation. This exonuclease deficient mutant, however, can bind DNA in the exonuclease active center and does have a residual amount of exonuclease activity, which is 1000-fold less than wild type T4 DNA polymerase (Reha-Krantz and Nonay, 1993). Exo$^-$ DNA polymerase can initiate the proofreading pathway but is unable to excise nucleotides. For instance, after Exo$^-$ incorporates a nucleotide the act of adding the next nucleotide will out compete removal of the nucleotide that has already been incorporated. Thus, if the ethyl phosphotriester hinders DNA replication, the Exo$^-$ mutant DNA polymerase will be able to bypass the lesion with more ease than the wild type DNA polymerase.

The DNA template used in this assay, PT1*, was constructed as described in "Materials and Methods". This 25-mer template contains a mix of ethyl phosphotriesters in either the R or the S conformation three nucleotides from the 5' end of the template strand. A 3'-phosphate was also present to protect the template DNA from degradation.

Figure 5: Primer extension assay with D112A/E114A exonuclease deficient and wild type T4 DNA polymerase using PT1* as a template. Panel A: Exonuclease deficient T4 DNA polymerase at 20 nM was incubated with either 1 nM unmodified (C) or phosphotriester-containing DNA (PT1*). Panel B: Wild type T4 DNA polymerase at 10 nM was incubated with either unmodified (C) or phosphotriester-containing DNA (PT1*). Both reactions were run in the presence of 5 μM dNTPs or 100 μM dNTPs and aliquots of these reactions were stopped at the time points located above each lane. Time points are in minutes with the exception of the 20- second (20") time point. The sequence of nucleotides added to extend the labelled primer is provided along with the number of incorporation events. For example, incorporation of the first nucleotide, an A, is denoted +1. The position of the ethyl phosphotriester is indicated by *. Any bands below the position of the primer correspond to degradation products.


A
Exonuclease-
5 uM
100uM
C
PT1*
C
PT1*
20" 2' 20" 2' 10' 20" 20"
Full length
+10
+9
+8
+7
+6
+5
+4
+3
+2
+1
Primer
A A A * A * C T A A A A
B
Wild-type
5 uM
100uM
C
PT1*
C PT1*
20" 2' 20" 1' 2' 10' 20" 20"

The primer, P1, was labelled at the 5' end with ^{32}P and annealed to PT1*. Reaction mixes contained 1 nM labelled primer-template and either 5 μM or 100 μM dNTPs. A low concentration (5 μM) of dNTPs was initially used in this assay to slow elongation. Under these experimental conditions, any effect that an ethyl phosphotriester may have on forming the Enzyme:DNA:dNTP complex required for elongation is enhanced. Reaction mixes containing either unmodified DNA (C) or phosphotriester DNA (PT1*) were started with 20 nM Exo$^-$ T4 DNA polymerase. The reaction products were separated and visualized by polyacrylamide gel electrophoresis on 20% acrylamide-7M urea gels.

Using the unmodified template (C), Exo$^-$ DNA polymerase completely extended most of the primer (full length) by 20 seconds (Figure 5, panel A). Faint bands corresponding to shorter than full length products were produced by a small fraction of incompletely deprotected template DNA or depurination/depyrimidination. The intensity of these bands did not change with time and, therefore, represent the background of the assay. In addition, the background level has been intensified using ImageQuant software to visualize these intermediate bands, since they can be used as a size ladder for extension products. This aids in observing the mobility of extended primer along with any bands that correspond with DNA polymerase pausing.

A band (Figure 5, panel A, +7 band) due to pronounced polymerase pausing was observed when Exo$^-$ T4 DNA polymerase replicated the DNA containing an ethyl phosphotriester (PT1*). The DNA polymerase paused at the

nucleotide immediately before the ethyl phosphotriester (Figure 5, panel A, 5 μM dNTP, 20s, +7 band). With time, however, Exo⁻ DNA polymerase was able to bypass the phosphotriester as shown by the reduction in intensity of this band at the 10-minute time point. At high dNTP concentration (100 μM), however, no significant differences were observed between the replication of unmodified and phosphotriester DNA.

B) *DNA replication by wild type T4 DNA polymerase is severely hindered by an ethyl phosphotriester in the DNA template in the presence of a low dNTP concentration.*

Wild type T4 DNA polymerase has a potent 3'→5' exonuclease activity (Table 3). Thus, by using wild type T4 DNA polymerase the effect of an ethyl phosphotriester on DNA replication in the presence of exonucleolytic proofreading can be observed. Wild type T4 DNA polymerase can perform the "idling" reaction, which involves the repeated incorporation and subsequent excision of a nucleotide (Hershfield and Nossal, 1972). Whether the newly incorporated nucleotide will be removed or whether the next nucleotide will be added depends upon the status of the primer-terminus. If elongation is slowed then wild type T4 DNA polymerase will have more opportunity to proofread. At a low dNTP concentration (5 μM), elongation by wild type T4 DNA polymerase is slowed and the polymerase is predicted to proofread, while the opposite is expected using a high dNTP concentration (100 μM) (Clayton *et al.*, 1979).

With the unmodified DNA template (C), mostly full length product was

produced with a significant amount of product one nucleotide short of full length (full length-1) (Figure 5, panel B, +9 band). At the end of a primer template, the number of protein-DNA contacts are much less because upstream template nucleotides are no longer present. Thus, DNA polymerases have more difficulty incorporating the last nucleotide at the end of a primer template than at earlier positions. The increased difficulty of replicating DNA to full length allows the DNA polymerase more opportunity to bind the DNA in the exonuclease active center. In this case, a DNA polymerase that is proficient in exonuclease activity will repeatedly excise and then incorporate the terminal nucleotide (idling). By using only 5 μM dNTPs, the inability of wild type T4 DNA polymerase to insert the last nucleotide or the opportunity for the polymerase to remove the last nucleotide is enhanced.

With phosphotriester DNA (PT1*), DNA replication by wild type T4 DNA polymerase was severely hindered. In Figure 5 (Panel B), hindrance of DNA replication was represented by the presence of a predominant pause band (+7 band) one nucleotide immediately before the ethyl phosphotriester. As reaction time increased from 20 seconds to 10 minutes, the +7 band appeared to decrease with the concomitant increase in full length (+10 band) and full length-1 (+9 band) product. At 10 minutes, however, the amount of full length product decreased along with the appearance of a second major band (+6 band) corresponding to a position two nucleotides before the ethyl phosphotriester. Since the dNTP concentration was only 5 μM it is likely that wild type T4 DNA polymerase exhausted the available nucleotide pool, resulting in degradation after 10 minutes.

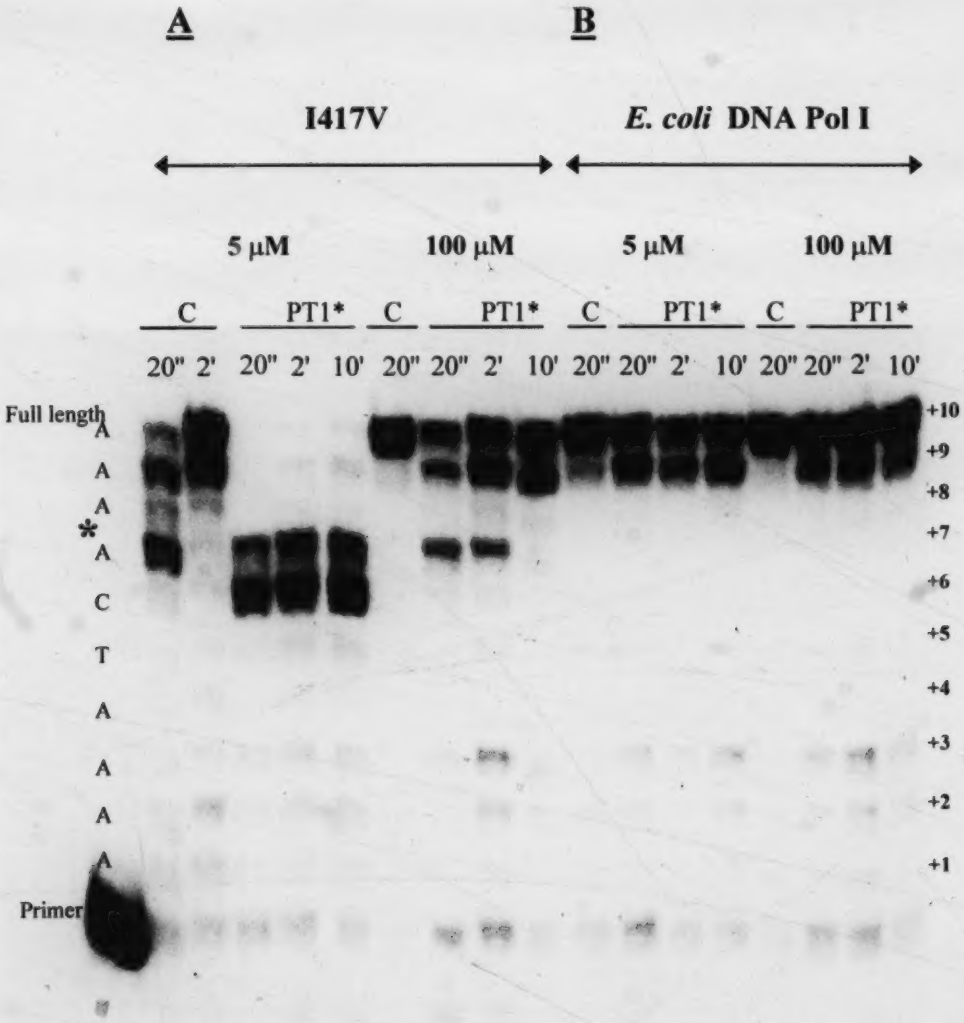
At 100 μM dNTPs, however, there was no significant difference between the replication of unmodified and phosphotriester DNA by wild type T4 DNA polymerase.

C) *DNA replication by I417V T4 DNA polymerase is completely blocked by an ethyl phosphotriester in the DNA template in the presence of a low concentration of dNTPs and is substantially slowed in the presence of a high concentration of dNTPs.*

The mutant, I417V-T4 DNA polymerase proofreads more often than wild type T4 DNA polymerase (Table 3). This is believed to be due to the reduced ability of the DNA polymerase to stabilize the primer terminus in the polymerase active center, thus allowing for a greater opportunity to proofread (Reha-Krantz and Nonay, 1994). With an increased opportunity to proofread, each time that I417V-T4 DNA polymerase inserts a nucleotide it is more likely than the wild type DNA polymerase to remove that nucleotide rather than incorporate the next nucleotide. Additionally, by using a low concentration of dNTPs elongation is further slowed and the effect of an ethyl phosphotriester should be enhanced.

The observations that an ethyl phosphotriester hindered DNA replication by wild type T4 DNA polymerase to a greater extent than Exo$^-$ T4 DNA polymerase, implied that the presence of 3'→5' exonucleolytic proofreading activity enhanced the inhibitory affect of this lesion. The I417V-T4 DNA polymerase has a reduced ability to bind DNA in the polymerase active center and,

Figure 6: Primer extension assay with I417V-T4 DNA polymerase and *Escherichia coli* DNA polymerase I using PT1* as a template. Panel A: I417V-T4 DNA polymerase at 10 nM was incubated with either unmodified (C) or phosphotriester-containing DNA (PT1*) at 1 nM. Panel B: *E. coli* DNA polymerase I at 10 nM was incubated with either unmodified (C) or phosphotriester-containing DNA (PT1*). Both reactions were run in the presence of 5 μM dNTPs or 100 μM dNTPs and aliquots of these reactions were stopped at the time points located above each lane. Time points are in minutes with the exception of the 20-second (20") time point. The sequence of nucleotides added to extend the labelled primer is provided along with the number of incorporation events (+1, +2….etc). The position of the ethyl phosphotriester is indicated by *.


A
B
I417V
E. coli DNA Pol I
5 μM
100 μM
5 μM
100 μM
C
PT1*
C
PT1*
C
PT1*
C
PT1*
20" 2' 20" 2' 10' 20" 20" 2' 10' 20" 20" 2' 10' 20" 20" 2' 10'
Full length
A
A
A
*
A
C
T
A
A
A
A
Primer
+10
+9
+8
+7
+6
+5
+4
+3
+2
+1

consequently, transfers the DNA from the polymerase active centre to the exonuclease active centre frequently (Reha-Krantz and Nonay, 1994). Therefore, it was predicted that DNA replication by I417V-T4 DNA polymerase would be inhibited to a greater extent by an ethyl phosphotriester than that observed for wild type DNA polymerase.

Using an unmodified template (C), at a low dNTP concentration (5 μM), I417V DNA polymerase had difficulty producing full length product in the first 20 seconds (Figure 6, panel A). This result confirms previous findings that I417V-T4 DNA polymerase is slower at replicating DNA than wild type T4 DNA polymerase (Reha-Krantz and Nonay, 1994). Within 2 minutes, however, all of the primer was extended to full length (+10 band) and full length-1 (+9 band) products.

In contrast, under similar reaction conditions, DNA replication by the I417V-T4 DNA polymerase was completely blocked by the presence of an ethyl phosphotriester in the primer template (Figure 6, panel A, 5 μM dNTPs). I417V DNA polymerase was blocked at two positions, which are located two nucleotides (+6 band) and one nucleotide (+7 band) before the ethyl phosphotriester in the DNA template. With increasing time the intensity of the bands corresponding to DNA replication blocks did not decrease, indicating that these bands represent substrates that could not be extended or degraded.

At high dNTP concentrations (100 μM) with the unmodified template, I417V DNA polymerase extended all of the primer to full length in 20 seconds (Figure 6, panel A). With the DNA substrate containing the ethyl phosphotriester,

however, a band corresponding to DNA polymerase pausing or degradation, was present one nucleotide before the ethyl phosphotriester (+7 band). This pause band was no longer present at the 10-minute time point indicating that I417V DNA polymerase eventually bypassed the ethyl phosphotriester. The presence of an ethyl phosphotriester also appeared to greatly enhance the production of full length-1 (+9 band), which comprised approximately 50% of all extension products, as determined by densitometry. Using the unmodified template, there appeared to be no full length-1 produced in the first 20 seconds.

D) *DNA replication by Escherichia coli DNA polymerase I is not hindered at the site of an ethyl phosphotriester in the DNA template.*

The effect of DNA containing ethyl phosphotriesters on DNA replication has been studied previously by Miller *et.* al (1982). This study found that an ethyl phosphotriester slowed DNA replication by *E. coli* DNA polymerase I (*E. coli* DNA pol I), and that the Rp isomer had a greater inhibitory effect than the Sp isomer. Thus, we tested *E. coli* DNA pol I under similar reaction conditions to confirm this result.

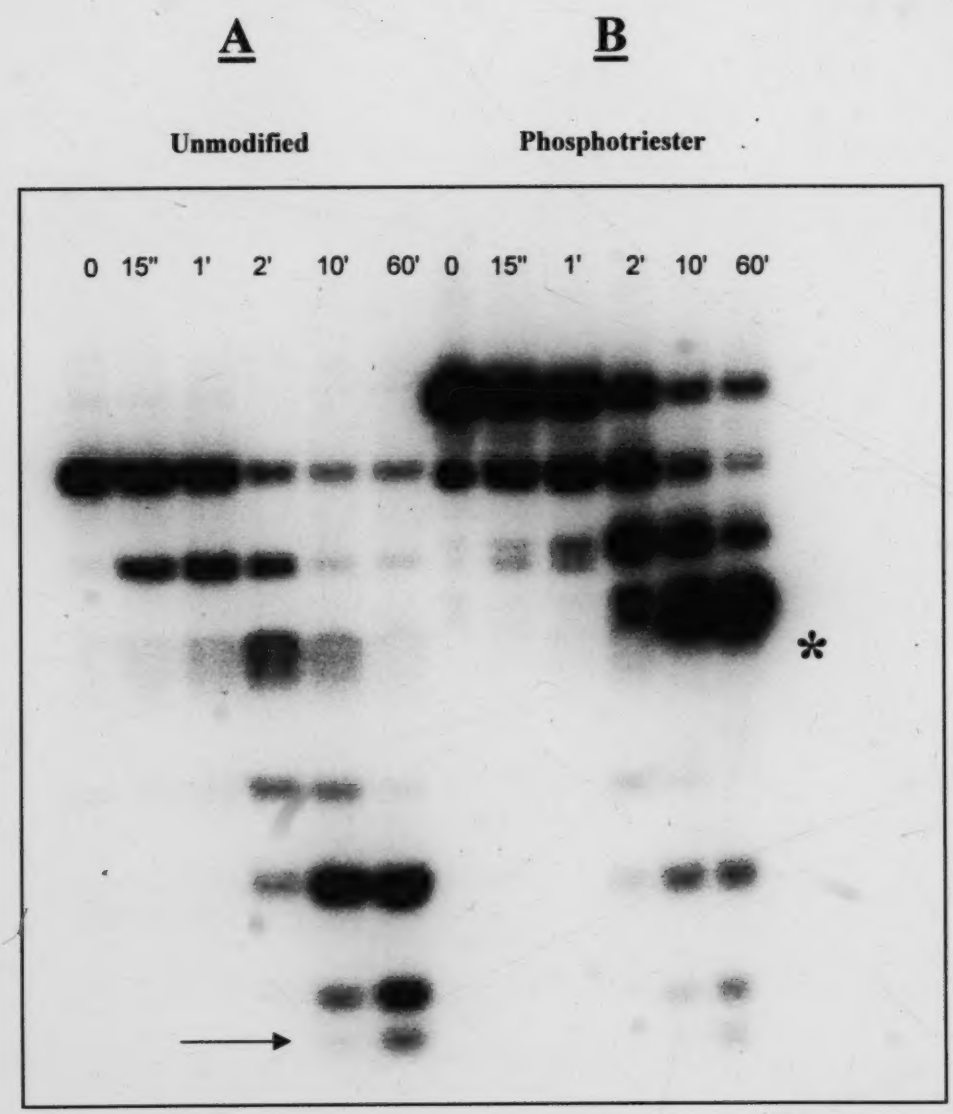
Using the unmodified template and 5 μM dNTPs, *E. coli* DNA pol I extended all of the primer to full length in 20 seconds (Figure 6, panel B). When phosphotriester DNA was replicated by *E. coli* DNA pol I, there were no pause bands detected. However, a substantial amount of full length-1 product (+9 band) was produced. Similar results were obtained when 100 μM dNTPs were used, although slightly more full length was made. Thus, DNA containing an ethyl

phosphotriester appeared to promote the production of full length-1 product (+9 band), by *E. coli* DNA pol I, irrespective of nucleotide concentration.

E) *An ethyl phosphotriester is still intact in 90% of the phosphotriester DNA sample (PT1).*

Over time, de-ethylation of phosphotriesters in the DNA backbone occurs. In order to ensure that the ethyl phosphotriester was intact, a single-stranded exonuclease assay was performed. Phosphotriesters are resistant to degradation by all exo- and endonucleases tested to date (Jensen and Reed, 1978; Miller *et al.*, 1971; Gallo *et al.*, 1986). Therefore, if the ethyl phosphotriester is still intact in the original 10-mer oligonucleotide, PT1, then wild type T4 DNA polymerase should be unable to degrade this DNA past the location of the phosphotriester and products less than a 4-mer oligonucleotide should not be observed. As expected, wild type T4 DNA polymerase degraded the unmodified DNA (C10) (Table 2) down to a dinucleotide within 1 hour (Figure 7, panel A, dinucleotide indicated by an arrow). The dinucleotide is the limit digest for wild type T4 DNA polymerase and most other DNA polymerases with 3'→5' exonuclease activity (Huang and Lehman, 1972). Wild type T4 DNA polymerase, however, was unable to degrade PT1 past the location of the ethyl phosphotriester with the exception of a small fraction of unmodified DNA in the sample (Figure 7, panel B). A large difference in the mobilities of the unmodified and phosphotriester DNA was observed. Due to the addition of an ethyl group and loss of a negative charge, the phosphotriester DNA runs much slower on a 20% polyacrylamide-7M urea gel. Although this

Figure 7: Single-stranded DNA exonuclease assay with wild type T4 DNA polymerase using unmodified DNA and PT1 as substrates. Panel A: Wild type T4 DNA polymerase at 0.1 nM was incubated with labelled unmodified DNA (C10) at 1 nM. Panel B: Wild type T4 DNA polymerase at 0.1 nM was incubated with labelled phosphotriester-containing DNA (PT1) at 1 nM. Both reactions were run in the absence of dNTPs and aliquots of the reactions were stopped at the time points indicated above each lane. The time points are in minutes with the exception of the 15-second (15") time point. DNA degraded down to the dinucleotide is indicated by an arrow and the position of the ethyl phosphotriester is indicated by *. Bands below the position of the ethyl phosphotriester correspond to unmodified DNA.


A
B
Unmodified
Phosphotriester
0 15" 1' 2' 10' 60' 0 15" 1' 2' 10' 60'
*

difference in mobility makes sequence deciphering difficult, it aids in determining the amount of unmodified DNA in the phosphotriester DNA sample. Bands below the ethyl phosphotriester block were observed (Figure 7, panel B bottom of gel). Since these bands have the same mobility as adjacent bands in the unmodified DNA (Figure 7, panel A bottom of gel), these bands most likely represent unmodified DNA in the phosphotriester DNA preparation that may have been produced by de-ethylation during storage (Bannon and Verly, 1972). The total amount of this unmodified DNA, however, only comprises approximately 10% of the total DNA, as determined by densitometry. Therefore, the ethyl phosphotriester is intact in 90% of the phosphotriester DNA sample.

Discussion: Part 1

In 1982, Miller *et al.* demonstrated that an ethyl phosphotriester in a DNA template slowed DNA replication by *E. coli* DNA polymerase I. Since that time, no additional studies of the effect of an ethyl phosphotriester on DNA replication have been published. Mammalian cells appear not to repair phosphotriesters (Bodell *et al.*, 1979; Engelse *et al.*, 1987; Engelse *et al.*, 1986), implying that replication bypass of phosphotriester adducts must occur. In *E. coli*, only the Sp isomer of a phosphotriester is repaired (Hamblin and Potter, 1985; Weinfeld *et al.*, 1985; Teo *et al.*, 1986), leaving the Rp isomer to be either replicated or tolerated by a lesion bypass process. Our hypothesis was that an ethyl phosphotriester would hinder DNA replication by T4 DNA polymerase to a greater extent than *E. coli* DNA polymerase I. Unlike *E. coli* DNA polymerase I, which is a repair polymerase, T4 DNA polymerase is responsible for high fidelity replication of the chromosome. This study clearly demonstrates that an ethyl phosphotriester in the DNA template can slow or even completely block T4 DNA polymerase replication. Slowed elongation by the ethyl phosphotriester increases the opportunity for 3'→5' exonucleolytic activity resulting in stalled replication at the site of the lesion.

Four important conclusions can be made from this study. First, an ethyl phosphotriester in the DNA template hinders wild type T4 DNA replication in the presence of a low dNTP concentration (Figure 5, panel B, 5 μM dNTPs). Second, exonuclease deficiency (Exo$^-$) increases translesion replication (Figure 5, panel A),

and third, reduced ability to bind DNA in the polymerase active centre (I417V) decreases translesion replication of ethyl phosphotriesters (Figure 6, panel A). Finally, DNA replication by *E. coli* DNA polymerase I was not paused at the site of the ethyl phosphotriester (Figure 6, panel B).

Results obtained by Miller *et al.* (1982) demonstrated that DNA replication by *E. coli* DNA polymerase I was only slightly affected by an ethyl phosphotriester in the DNA template. According to Miller's results, the rates and extents of polymerization with DNA templates containing an ethyl phosphotriester were 25% (Sp) and 50% (Rp) less than with an unmodified DNA template. This data was obtained using an assay where the amount of radioactive guanine incorporated was quantified with a template that contained two cytosines at the 5'-terminus (Figure 4b). Thus, the incorporation of radioactive guanine was indicative of the production of full length and full length-1 products. With our *in vitro* primer extension assay we were able to observe that the presence of an ethyl phosphotriester did not block or pause the polymerase at the site of the lesion but instead, increased the ratio of full length-1 (+9 band) to full length product (Figure 6, panel B). Our results may parallel those obtained by Miller *et al.* (1982), in that DNA replication by *E. coli* DNA pol I is affected to different degrees with the Rp and Sp isomers of the ethyl phosphotriester. For instance, the full length-1 band (+9) may represent the replication of DNA containing the Rp isomer, while the full length band may represent the replication of DNA containing the Sp isomer (Figure 6, panel B). However, this would imply that the Rp ethyl phosphotriester is a 100% block to DNA replication, while the Sp isomer is not a block to replication

at all. A more likely scenario would be that replication of DNA containing the Rp isomer would result in a lesser amount of full length and, therefore, a greater amount of full length-1 than replication of the Sp isomer. In Miller's assay the ethyl phosphotriester was located five nucleotides from the 5' end of the template (Figure 4b) and in our assay the ethyl phosphotriester was located 3 nucleotides from the 5' end (Figure 4a). Therefore, the Rp and Sp isomers may have affected the manipulation (ie. bending) of the template, to differing extents, by *E. coli* DNA pol I when it attempted to incorporate a nucleotide at the terminal template position.

Because the template used in our assay contains four terminal thymines (PT1*, see Table 2), it is possible that an ethyl phosphotriester enhanced "breathing". DNA that is AT-rich has a relatively low melting temperature and tends to separate or "breathe" frequently (Brahms *et al.*, 1976). As DNA is replicated, the strands are often separated to allow for proofreading. Strand separation through proofreading is further facilitated by AT-rich DNA (Bessman and Reha-Krantz, 1977). *E. coli* DNA polymerase I, however, has a 1000-fold less 3'→5' exonuclease activity than wild type T4 DNA polymerase (Kornberg, 1980). Therefore, it is unlikely that the abundant production of full length-1 was due to the repeated excision and incorporation of the terminal nucleotide. Instead, it is more likely that *E. coli* DNA polymerase I dissociated from the DNA frequently at this position since this enzyme tends to dissociate often when it attempts to proofread (Joyce, 1989). Thus, an ethyl phosphotriester, in the DNA template, two nucleotides away from the primer terminus, may have induced dissociation or

inhibited rebinding of the polymerase. It is known that *E. coli* DNA pol I requires the melting out of 4-5 nucleotides of the primer in order to excise the terminal nucleotide (Cowart *et al.*, 1989). Because the incorporation of the terminal nucleotide must occur without the presence of downstream contact sites, polymerases may first "choose" to bind in a mode suited for exonuclease activity. Therefore, at the terminal template position, *E. coli* DNA pol I may be attempting to bind DNA in the exonuclease active centre more frequently than in the polymerase active centre. Since *E. coli* DNA pol I often dissociates upon attempting to switch from the polymerase to the exonuclease active center, the loss of an important phosphate contact site could have enhanced this dissociation.

A second possibility is that the incorporation of a nucleotide, by *E. coli* DNA pol I, opposite the terminal template nucleotide was affected by the presence of an ethyl phosphotriester two nucleotides downstream. Through studies using a primer-template containing a bulky probe at various positions, it was discovered that the exonuclease activity of Klenow fragment (the large fragment of *E. coli* DNA pol I) was inhibited when the probe was 15 nucleotides downstream from the primer terminus. In contrast, polymerization was inhibited when the probe was six nucleotides downstream from the primer terminus (Cowart *et al.*, 1989; Gopalakrishnan and Benkovic, 1994). Thus, it is possible that an ethyl phosphotriester two nucleotides downstream affected the incorporation of a nucleotide by *E. coli* DNA polymerase I, rather than promoting the removal of an already incorporated nucleotide.

Wild type and I417V-T4 DNA polymerase produced significantly more full length-1 than *E. coli* DNA polymerase I when replicating phosphotriester DNA (Figure 5, panel B and Figure 6, panels A and B). Both wild type and I417V-T4 DNA polymerases contain potent 3'→5' exonuclease activities, and therefore, the frequency that proofreading and subsequently strand separation will occur is very high. The finding that a significant amount of full length-1 was produced with the unmodified template only when nucleotides were limiting, suggests that nucleotide incorporation at the terminal template position was decreased because wild type and I417V-T4 DNA polymerases exhausted the pool of dATP. As previously mentioned, the template used in this assay contained four terminal thymines and the ethyl phosphotriester was located three nucleotides from the 5'-terminus. Wild type and I417V-T4 DNA polymerase may have repeatedly incorporated and then excised adenine residues at the site of the ethyl phosphotriester, resulting in the depletion of dATP. Even with an originally high concentration of dNTPs, an ethyl phosphotriester could have prolonged DNA polymerase "idling" (Figure 8) enough to deplete dATP to an extent that mimics assays performed with a low concentration of dNTPs. The finding that the I417V DNA polymerase produced similar amounts of full length-1 with phosphotriester DNA at 100 μM dNTPs as it does with unmodified DNA at 5 μM dNTPs (Figure 6, panel A) further supports our model that nucleotides are being "wasted" at the site of the ethyl phosphotriester.

Replication bypass of an ethyl phosphotriester in the DNA template by wild type, D112A/E114A and I417V-T4 DNA polymerase was hindered under

reaction conditions containing low dNTP concentrations (Figures 5 and 6). Reducing the amount of available dNTPs impedes the formation of the Enzyme:DNA:dNTP ternary complex required for nucleotide incorporation, resulting in slowed elongation. Elongation is further slowed by the presence of an ethyl phosphotriester, suggesting that the lesion interferes with the formation of the ternary complex. If the formation of the ternary complex is difficult, then the DNA is transferred from the polymerase to the exonuclease active site, which results in nucleotide excision by those DNA polymerases that are proficient in 3'→5' exonuclease activity (Baker and Reha-Krantz, 1998). In accordance with this, the I417V DNA polymerase, which has a reduced ability to form the ternary complex and a proficient 3'→5' exonuclease activity, was inhibited to the greatest extent by an ethyl phosphotriester.

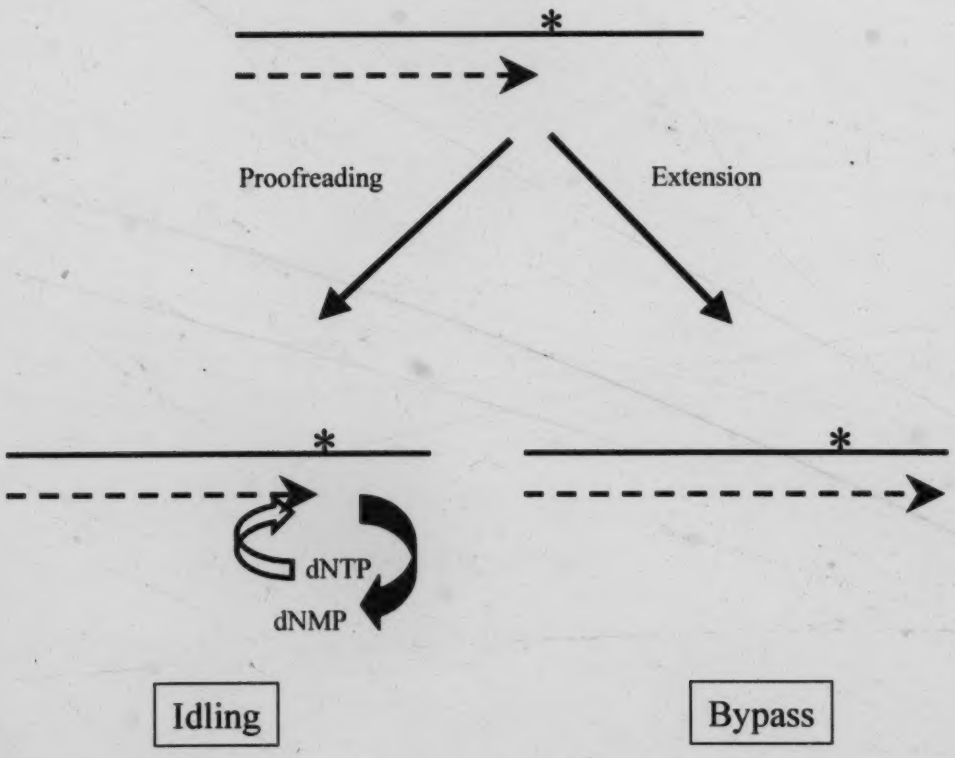
Our observations suggest that an ethyl phosphotriester pauses DNA replication and prolongs "idling" by exonuclease proficient T4 DNA polymerases. For instance, elongation by wild type, Exo$^-$ and I417V-T4 DNA polymerases was slowed by the presence of the ethyl phosphotriester, resulting in the transfer of DNA from the polymerase to the exonuclease active site. Exo$^-$ DNA polymerase is unable to excise nucleotides and, therefore, this polymerase is able to dissociate and rebind the primer template without being sequestered in a non- productive idling reaction. In addition, when Exo$^-$ DNA polymerase incorporates a nucleotide across from the template base immediately after the ethyl phosphotriester it is unable to remove it. In contrast, wild type and I417V DNA polymerase are able to excise nucleotides, resulting in the repeated excision of a nucleotide and

subsequent incorporation of another at the site of the ethyl phosphotriester. This model (Figure 8) is supported by the finding that as the opportunity for proofreading is increased, from T4 DNA polymerases ranging from exonuclease-deficient to wild type to above wild type levels, so did the inhibitory effect of the ethyl phosphotriester. Thus, an ethyl phosphotriester may promote the depletion of nucleotide pools *in vivo* by slowing elongation and prolonging the idling reaction. Although nucleotides are recycled *in vivo* and are thought to be concentrated at replication sites (Mathews and Sinha, 1982), it was observed that the limiting nucleotide in the mammalian nucleus is dGTP at 10 μM (Leeds *et al.*, 1985). The concentration of available dGTP in the nucleus is close to the low concentration (5 μM) of nucleotides used in our assays. Thus, data obtained from our assays at 5 μM dNTPs may reflect the effect of an ethyl phosphotriester located by template cytosines on DNA replication *in vivo*. These results suggest that DNA containing an ethyl phosphotriester may not be efficiently replicated in cells if the DNA polymerase that replicates the chromosome contains a potent exonuclease activity. However, it is unlikely that a replicative DNA polymerase would spend a lengthy time "idling" at the site of the ethyl phosphotriester without dissociating and initiating replication downstream or signalling the aid of enzymes involved in repair or bypass of the lesion.

The finding that an ethyl phosphotriester slows elongation has significant biological implications. For instance, stalled DNA replication may result in the recruitment of bypass polymerases to circumvent the lesion. In addition, tolerance through recombination may also occur. Either way ethyl phosphotriesters must be

Figure 8: DNA Polymerase Idling Model.

Each time that a DNA polymerase incorporates a nucleotide it can either incorporate another nucleotide (extension) or remove the already incorporated nucleotide (proofreading). Elongation may be slowed by the presence of DNA damage and slowed elongation results in proofreading. DNA polymerase idling occurs when the DNA polymerase repeatedly incorporates and then excises a nucleotide.



tolerated by some mechanism since they appear to remain unrepaired in the genome of mammalian cells. Localized repair of ethyl phosphotriesters at sites of transcription, however, may occur. This event is likely since ethyl phosphotriesters inhibit RNA polymerase binding at promoters (Siebenlist and Gilbert, 1980; Jorgensen *et al.*, 1991) and slow transcription *in vivo* (Marushige and Marushige, 1983). Transcription-blocking DNA damage that is not efficiently repaired by global repair mechanisms is efficiently repaired in transcriptionally active genes. The finding that pyrimidine dimers, a transcription-blocking lesion (Sauerbier and Hercules, 1978), are efficiently repaired in transcriptionally active genes (Bohr *et al.*, 1986) resolved the anomaly that while rodent and human cells survive equally well after UV-irraditation, they differ dramatically in their global repair of UV damage (Fujiwara and Tatsumi, 1976). For instance, the majority of pyrimidine dimers are repaired in human cells within a 24-hour period, whereas rodent cells remove only 15% of pyrimidine dimers in the same amount of time (Zelle *et al.*, 1980). Therefore, pyrimidine dimers appear to remain unrepaired in the overall genome of rodent cells. Like pyrimidine dimers, ethyl phosphotriesters also appear to evade global repair in mammalian cells, but are likely to be repaired in transcriptionally active genes by a similar mechanism.

Preface to Results: Part 2

The next set of experiments describe the replication of another phosphotriester-containing DNA substrate. We discovered late in our studies, however, that this DNA also contains acetyl groups on many guanine residues at the N^2-position. During the chemical synthesis procedure, isopropylphenoxyacetyl guanine-protecting groups, which are easily removed by deprotection with ammonia, were replaced by stable acetyl groups. Thus, acetyl groups on one or more guanine residues were present in the majority of DNA substrates used in this part. The N^2-acetylguanines block DNA replication to a much greater extent than the phosphotriester.

Results Part2: The effect of an ethyl phosphotriester on DNA replication using PT2 as a template.

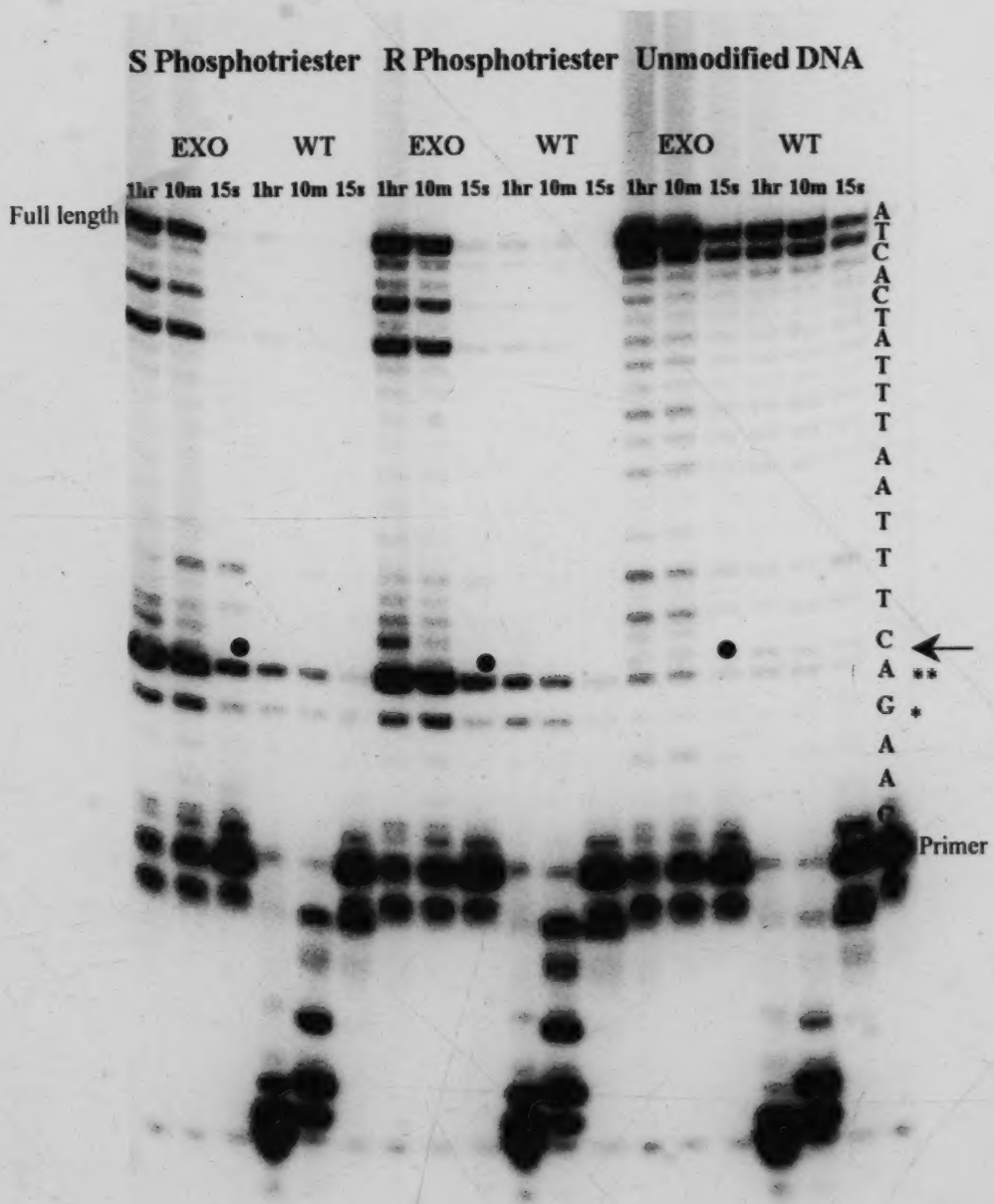
A) *Primer extension assay with wild type and D112A/E114A exonuclease deficient T4 DNA polymerase using PT2R, PT2S, and C1 as templates.*

The 32-mer oligonucleotides, PT2R and PT2S, are identical in sequence composition but contain an ethyl phosphotriester in the Rp and Sp conformation, respectively (Table 2). These oligonucleotides were synthesized as described in Materials and Methods and were deprotected in concentrated aqueous ammonia for 20 minutes at 65 °C. In initial experiments the unmodified template used, C1 (Table 2), was synthesized via conventional solid-state phosphoramidite chemistry by the Department of Biological Sciences.

The 11-mer primer, P2, was labelled at the 5' end with ^{32}P and annealed to PT2R, PT2S, or the unmodified template (C1). Reactions contained 1 nM labelled primer-template, 100 μM dNTPs, and 1 nM wild type or 2 nM Exo$^-$ T4 DNA polymerase. The reactions were stopped at various time points and reaction products were separated by gel electrophoresis on a 15% polyacrylamide-7M urea gel.

With the unmodified template, C1, wild type T4 DNA polymerase extended most of the primer to full length product within 1 hour (Figure 9, panel A). There was, however, a large amount of degradation product (bands below primer) also produced. This probably indicates that all of the primer was not annealed and was therefore degraded by wild type T4 DNA polymerase.

Figure 9: Primer extension assay with wild type and D112A/E114A exonuclease deficient T4 DNA polymerase using C1, PT2R, and PT2S as templates. Panel A: Wild type T4 DNA polymerase at 1 nM was incubated with unmodified DNA (C1) at 1 nM. Panel B: Exonuclease deficient T4 DNA polymerase at 2 nM was incubated with unmodified DNA (C1) at 1 nM. Panel C: Wild type T4 DNA polymerase incubated with PT2R. Panel D: Exonuclease deficient T4 DNA polymerase incubated with PT2R. Panel E: Wild type T4 DNA polymerase incubated with PT2S. Panel F: Exonuclease deficient T4 DNA polymerase incubated with PT2S. All reactions were run in the presence of all four dNTPs and aliquots of the reactions were stopped at the time points located above each lane. The sequence of nucleotides added to extend the labelled primer is indicated on the right adjacent to the corresponding band. Any bands below the position of the primer represent degradation products. The position of the ethyl phosphotriester is indicated by an arrow. The band corresponding to blocked DNA replication immediately before the ethyl phosphotriester is indicated by **, whereas the band corresponding to a block two nucleotides before the ethyl phosphotriester is indicated by *. A closed circle indicates the misincorporation band.


F E D C B A
S Phosphotriester
R Phosphotriester
Unmodified DNA
EXO WT EXO WT EXO WT
1hr 10m 15s 1hr 10m 15s 1hr 10m 15s 1hr 10m 15s 1hr 10m 15s 1hr 10m 15s
Full length
ATCACTATTTTAATTTTCAGAAG
Primer

Intermediate bands were observed and, as stated in the previous section (Part 1), these bands represent blocked DNA replication by not fully deprotected oligonucleotides produced during the chemical synthesis procedure or by depurination/depyrimidination. Two closely running bands can be observed at positions five and six above the primer band (Figure 9, bands adjacent to C and A in ladder). Bowling *et al.*, (1991) showed that oligonucleotides with the same sequence, except for the 3' terminal nucleotide, have different mobilities. Thus, an oligonucleotide ending in a C will run faster than the same oligonucleotide ending in an A. An oligonucleotide ending in an A will run faster than an oligonucleotide ending in a G, whereas an oligonucleotide ending in a T runs the slowest. Thus, the close spacing observed between the bands corresponding to a C and an A at the 3' terminal position is due to this difference in mobility.

With Exo$^-$ T4 DNA polymerase and unmodified DNA most of the primer has been extended to full length and full length + 1 nucleotide products within one hour (Figure 9, panel B). Other exonuclease deficient DNA polymerases, such as Exo$^-$ Klenow Fragment (Clark *et al.*, 1987) and *Thermus aquaticus* (Taq) (Clark, 1988), are also known to add a terminal non-templated nucleotide following full length replication.

A small amount of extension product may represent a misincorporation event (Figure 9, indicated by a closed circle). This band runs between the band corresponding to a correctly incorporated C and the band corresponding to a correctly incorporated T. This misincorporation band was not present when wild

type T4 DNA polymerase was used and is therefore, intrinsic to Exo⁻ T4 DNA polymerase.

Using PT2R as a template, wild type DNA polymerase produced little full length product and the polymerase appeared to be blocked at two major positions (Figure 9, panel C). The first block was located two nucleotides before the Rp-ethyl phosphotriester (block indicated by *, phosphotriester indicated by the arrow) while the second block was located at the nucleotide immediately before the Rp-ethyl phosphotriester (indicated by **). Interestingly, the majority of the DNA appeared to be degraded (bands below primer).

Exo⁻ T4 DNA polymerase was able to produce more full length product than wild type T4 DNA polymerase but replication appeared to be blocked at several locations (Figure 9, panel D). DNA replication by Exo⁻ DNA polymerase was blocked at the same positions that wild type DNA polymerase was blocked at (indicated by * and **). Exo⁻ DNA polymerase was also blocked at three and five bands below the full length band (-3 and –5). The intensity of these bands did not decrease as reaction time increased, indicating that these substrates could not be extended by Exo⁻ DNA polymerase. DNA replication blocks at the –3 and –5 positions both occur at nucleotides before template guanosines. The possible misincorporation band, produced with the unmodified DNA template, appeared to be enhanced by the presence of an Rp-ethyl phosphotriester in the template (Figure 9, panel D, closed circle).

Similar results were observed with both wild type and Exo⁻ T4 DNA polymerases when PT2S was used as a template (Figure 9, Panels E and F). With

Exo$^-$ T4 DNA polymerase and a DNA template containing an Sp-ethyl phosphotriester, DNA replication was blocked 2 nucleotides and 1 nucleotide before the S-ethyl phosphotriester, and at the –3 and –5 bands (Figure 9, panel F).

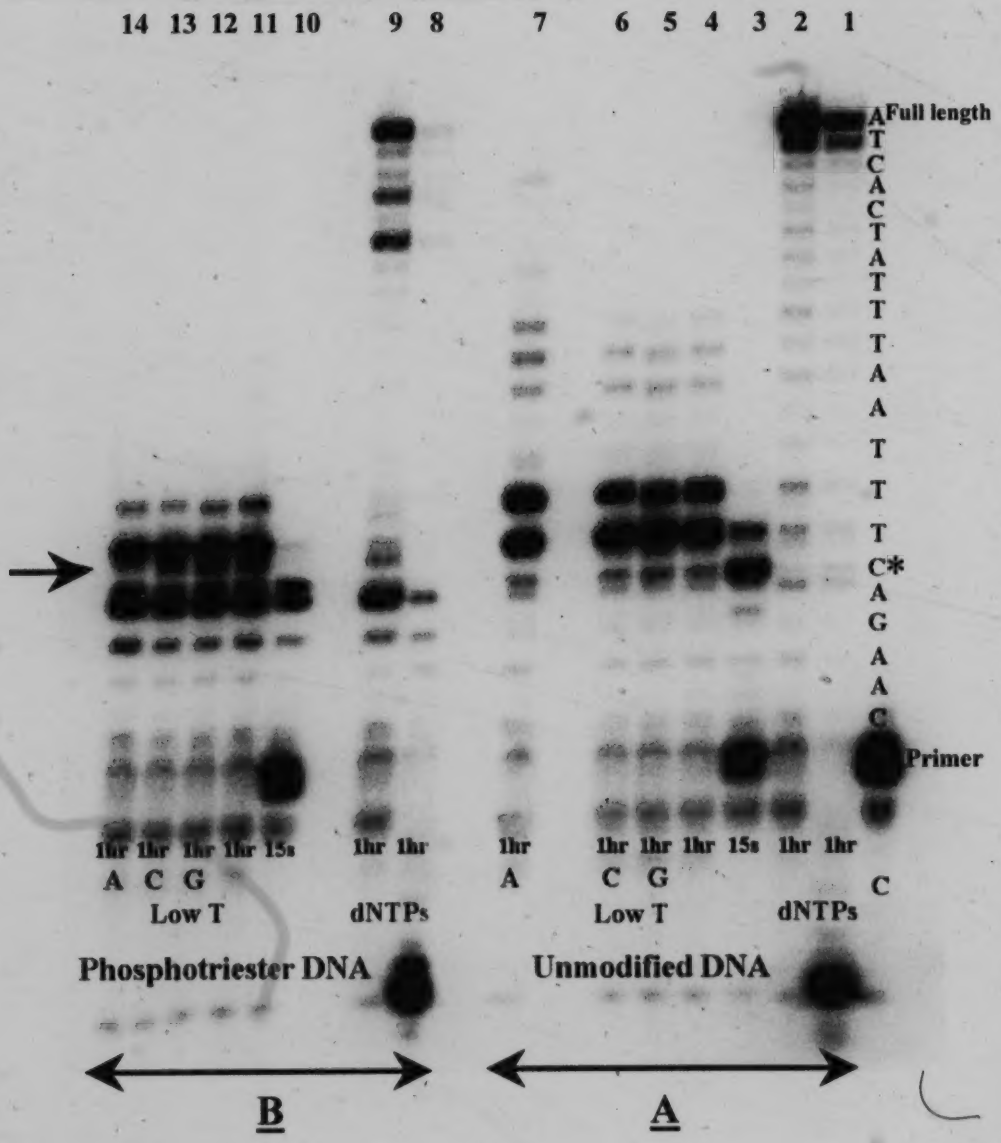
These results indicate that more than one kind of DNA template was present in the synthesized phosphotriester DNA samples. A small amount of unmodified DNA was expected to be present in the phosphotriester DNA samples. Unmodified DNA should be replicated faster than modified DNA. Therefore, we predicted that the amount of full length product produced in 15 seconds by Exo$^-$ T4 DNA polymerase would represent the amount of unmodified DNA in the phosphotriester DNA sample. If both unmodified and phosphotriester containing DNA are in the sample, then two types of reaction kinetics are expected. There were, however, at least three complex replication kinetics including a slow and a fast reaction rate. For instance, there appeared to be four blocks to DNA replication by Exo$^-$, located two nucleotides (*) and one nucleotide (**) before the ethyl phosphotriester and at the –3 and –5 positions. However, the intensity of the full length band increased significantly in 10-minutes, indicating that a proportion of the DNA did not completely block the polymerase but was not replicated as fast as unmodified DNA. In addition, there was also the presence of a possible misincorporation band (Figure 9, indicated by a closed circle) that increased in intensity as reaction time increased.

B) *Primer extension assay with wild type and D112A/E114A exonuclease deficient T4 DNA polymerase using differing concentrations of each dNTP and low dTTP.*

To determine what nucleotide was being incorporated to produce the misincorporation band, primer extension assays were performed with approximately 0.1 μM dTTP, and either 5 μM dATP, dGTP, or dCTP. An A is located in the template two nucleotides after the ethyl phosphotriester (Table 2) requiring the incorporation of a T at the complementary position. By using greatly reduced dTTP concentration the extension products below this position will build up. With the exception of dTTP and the desired dNTP at a concentration of 5 μM, all other dNTPs were added so that their final concentration was 100 μM.

Our prediction was that the location of the misincorporation event occurred at the guanine adjacent to the ethyl phosphotriester. As previously mentioned, a C at the 3' end of an oligonucleotide results in an oligonucleotide with a fast mobility on a polyacrylamide gel relative to this oligonucleotide ending in another dNTP (Bowling *et al.*, 1991). Conversely, with a T at the 3' end of the oligonucleotide, mobility on a polyacrylamide gel is relatively slow. The misincorporation band (indicated by the arrow) is located above the band corresponding to the extended primer with a correctly incorporated C (band above the arrow), indicating that another nucleotide besides C may have been added. Also, the misincorporation band runs below the band corresponding to the extended primer with a correctly incorporated T (band above the arrow). This implies that if the misincorporation

Figure 10: Primer extension assay with wild type and D112A/E114A exonuclease deficient T4 DNA polymerase using differing concentrations of each dNTP and low dTTP. Panel A: Assays with the unmodified DNA template (C1) at 1 nM. Panel B: Assays with the phosphotriester-containing DNA template, PT2R at 1 nM. Lane 1: Assay with wild type T4 DNA polymerase at 1 nM and all 4 dNTPs at 100 μM. Lane 2: Assay with exonuclease deficient T4 DNA polymerase (Exo$^-$) at 1 nM and all 4 dNTPs at 100 μM. Lanes 3 and 4: Assay with 0.1 μM dTTP, 100 μM dATP, dCTP, and dGTP, and Exo$^-$ DNA polymerase. Lanes 5 and 12: Assay with 0.1 μM dTTP, 5 μM dGTP, 100 μM dATP and dCTP and Exo$^-$. Lanes 6 and 13: Assay with 0.1 μM dTTP, 5 μM dCTP, 100 μM dATP and dGTP and Exo$^-$ DNA polymerase. Lanes 7 and 14: Assay with 0.1 μM dTTP, 5 μM dATP, 100 μM dCTP and dGTP and Exo$^-$ DNA polymerase. Lane 8: Assay with wild type T4 DNA polymerase and all 4 dNTPs at 100 μM. Lane 9: Assay with Exo$^-$ DNA polymerase and all 4 dNTPs at 100 μM. Lanes 10 and 11: Assay with 0.1 μM dTTP, 100 μM dATP, dCTP, and dGTP, and Exo$^-$ DNA polymerase. Reactions were stopped at the time points specified under each lane. The sequence of nucleotides added to extend the primer is provided on the right of the gel, along with the position of the ethyl phosphotriester which is indicated by *. The position of the misincorporation band is indicated by an arrow. All bands below the position of the primer band represent degradation products.


14 13 12 11 10 9 8 7 6 5 4 3 2 1
Full length
ATCACTATTTTAATTTC*AGAAC
Primer
1hr 1hr 1hr 1hr 15s 1hr 1hr
A C G Low T dNTPs
Phosphotriester DNA
1hr 1hr 1hr 1hr 15s 1hr 1hr
A C G Low T dNTPs C
Unmodified DNA
B
A

event occurred at this site then the misincorporated base should be an A, C or G residue.

The exact nucleotide that is being misincorporated at either position can be predicted by sequentially reducing the concentration of each dNTP. For instance, if dATP is being misincorporated then as the concentration of dATP is reduced the intensity of the misincorporation band should also decrease. In addition, by lowering each dNTP the location of the misincorporation event can be predicted. If the intensity of the misincorporation band decreases when dATP is low, then A may have been incorporated either opposite the template G that is immediately after the ethyl phosphotriester or the template A residue that is two nucleotides after the ethyl phosphotriester. A misincorporated dGTP at either template position could also produce the misincorporation band. However, if the intensity of the misincorporation band decreases when dCTP is low, then C must have been incorporated opposite the template T that is located two nucleotides after the ethyl phosphotriester.

The primer P1 was labelled at the 5' end with ^{32}P and annealed to C1 or PT2R. Reactions were started with 1 nM wild type T4 DNA polymerase or 2 nM Exo$^-$ T4 DNA polymerase and stopped at a reaction time of 15 seconds and 60-minutes. Wild type DNA polymerase was used in this experiment to produce a ladder with the unmodified template and the template containing the ethyl phosphotriester.

Using the unmodified DNA template (C1) and low dTTP, Exo$^-$ DNA

polymerase was blocked at the first and second positions requiring the incorporation of dTTP (Figure 10, panel A lane 4). As previously mentioned, the misincorporation band was produced using the unmodified DNA template but in very low amounts (Figure 10, panel A position of arrow), making it difficult to determine if the amount of misincorporation product decreased when using lower concentrations of dATP, dCTP, or dGTP.

Using phosphotriester DNA template and a low concentration of dTTP, Exo$^-$ T4 DNA polymerase was primarily blocked at the position immediately before the ethyl phosphotriester, the nucleotide immediately after the ethyl phosphotriester, the proposed misincorporation site, and the first position in the template coding for T (Figure 10, panel B, lane 11). The intensity of the misincorporation band (indicated by the arrow) decreased significantly when the concentration of dATP was low (Figure 10, panel B, lane 14). Additionally, when the concentration of dCTP was low the intensity of the misincorporation band also decreased, but to a lesser extent (Figure 10, panel B, lane 13). In assays containing only low dTTP, the intensity of the misincorporation band was very high and did not change when the concentration of dGTP was reduced (Figure 10, panel B, lanes 11 and 12).

These results suggest four possibilities for the observed misincorporation. First, dCTP is being misincorporated opposite template A, which was located two nucleotides after the ethyl phosphotriester or second, dATP was misincorporated at the same position. A third possibility is that dATP was misincorporated opposite template G immediately 5' to the ethyl phosphotriester. Finally, a combination of

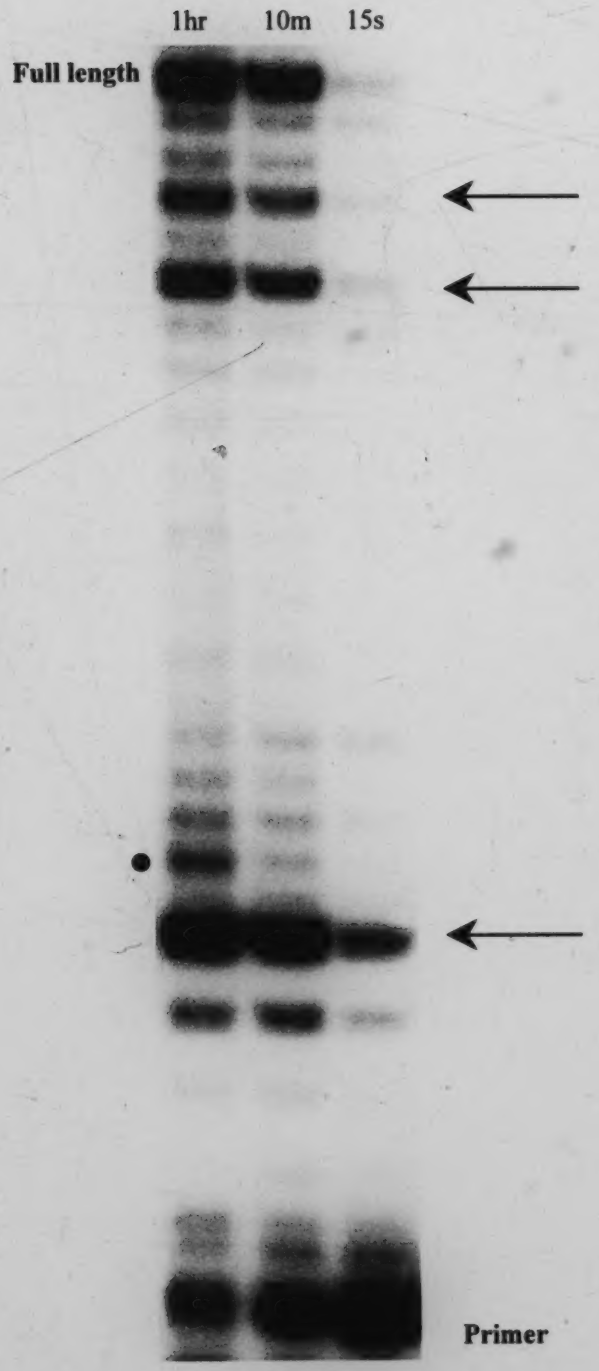
both misincorporation events could have lead to the production of the misincorporation band.

C) *Primer extension assay with D112A/E114A exonuclease deficient T4 DNA polymerase using C2 as a template.*

The unmodified template, C1, was synthesized by a different method than the phosphotriester templates, PT2R and PT2S (see Materials and Methods). Due to the possibility of more than two types of DNA (i.e. unmodified DNA and DNA containing an ethyl phosphotriester) in the phosphotriester DNA sample, a second unmodified template was synthesized since different synthetic methods may result in different DNAs. The unmodified template, C2, was synthesized by the same method as the phosphotriester DNA templates and was deprotected with concentrated aqueous ammonia for 20 minutes at 65 °C. This DNA was used to determine if the blocks, pause bands, and the misincorporation band are due solely to the ethyl phosphotriester or another adduct produced during the synthesis procedure.

In Figure 11 Exo$^-$ T4 DNA polymerase was paused at three distinct sites (indicated by arrows) on the control DNA template C2. All three of these bands corresponding to DNA replication blockage, are located at the nucleotide immediately preceding a guanosine in the template strand (Table 2). In addition, the misincorporation band (Figure 11, indicated by a closed circle) is still being produced. These findings indicate a modification of the guanine residues in the DNA template may have occurred. Since this control DNA template, C2, was

Figure 11: Primer extension assay with D112A/E114A exonuclease deficient T4 DNA polymerase using C2 as a template. Exonuclease deficient D112A/E114A-T4 DNA polymerase at 2 nM was incubated with C2 annealed to a labelled primer (primer template at 1 nM) in the presence of all four dNTPs. Aliquots of the reaction were stopped at the time points located above each lane. Bands corresponding to DNA replication blocks are indicated by arrows, while the misincorporation band is indicated by a closed circle.

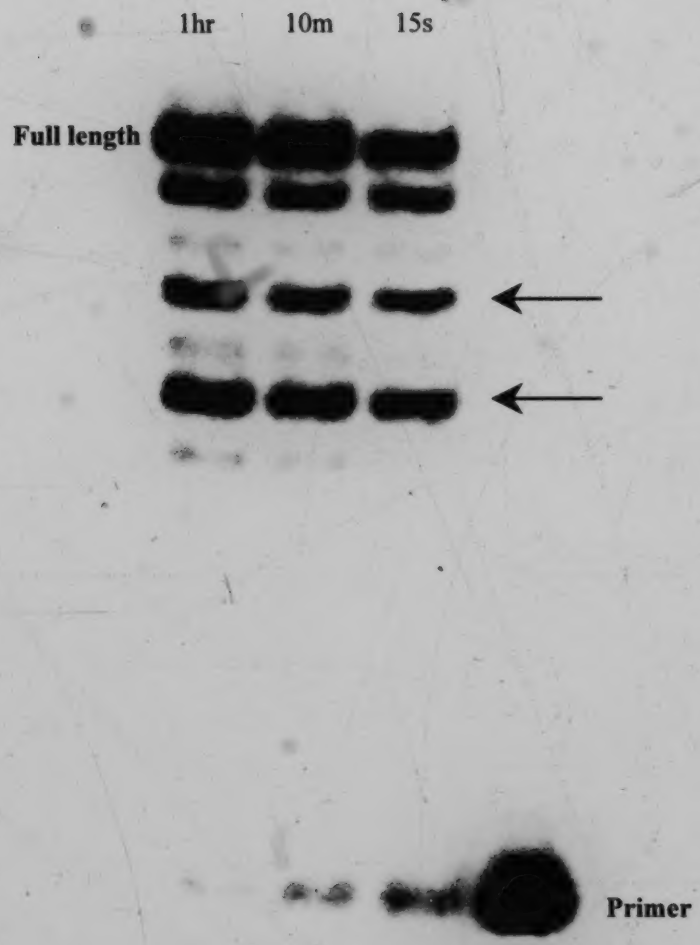

1hr
10m
15s
Full length
Primer

synthesized by the same method as PT2R and PT2S then the guanine residues in these templates may also be modified. In addition, the ethyl phosphotriester in PT2R and PT2S is located in between a guanine and a thymine. Therefore, DNA replication blocks observed at this position may be due a modified guanine. Also, the misincorporation band is still being produced implying that the misincorporation event is not likely due to an ethyl phosphotriester, in the DNA template.

D) *Primer extension assay with D112A/E114A exonuclease deficient T4 DNA polymerase using PT2R as a template and P3 as a primer.*

Using the unmodified template, C2, DNA replication by Exo$^-$ T4 DNA polymerase was blocked at template guanines. Replication blocks at the –3 and –5 positions were observed using C2, PT2R, and PT2S. Therefore, it is unlikely that the –3 and –5 bands were due to the ethyl phosphotriester but to confirm this, a different primer was used. The 20-mer primer, P3, anneals to PT2R so that its 3'-end is now four nucleotides past the ethyl phosphotriester (Table 2). If the –3 and –5 bands were due to the ethyl phosphotriester, then they should disappear or significantly reduce in intensity when using this primer. On the other hand, the –3 and –5 bands will remain if they are due to a modification of the guanine bases. Reactions consisted of 1 nM labelled primer-template, 100 μM dNTPs, and 2 nM Exo$^-$ T4 DNA polymerase. At various time points aliquots of the reaction mix were stopped and separated by gel electrophoresis on a 15% polyacrylamide-7M urea gel.

Figure 12: Primer extension assay with D112A/E114A exonuclease deficient T4 DNA polymerase using PT2R as a template and P3 as a primer. Labelled P3 primer anneals to PT2R so that it's 3'-end is four nucleotides past the position of the ethyl phosphotriester. This labelled primer template substrate (1 nM) was incubated with Exo$^-$ DNA polymerase at 2 nM and aliquots of the reaction were stopped at the time points located above each lane. Bands representing DNA replication blocks at the –3 and –5 positions are indicated by arrows.


1hr
10m
15s
Full length
Primer

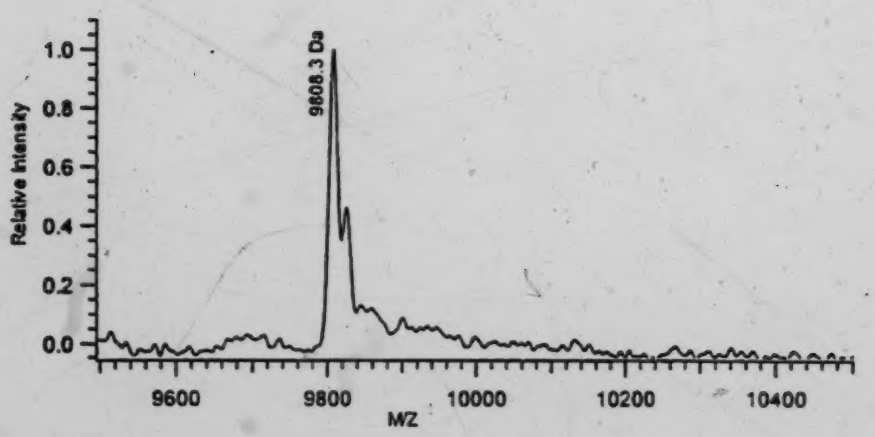
Using the elongated P3 primer, the –3 and –5 bands were still present (Figure 12, -3 and –5 bands indicated by arrows). The intensity of these bands did not change with increasing reaction time, indicating that Exo⁻ DNA polymerase was unable to extend these substrates. Thus, the –3 and –5 bands were not due to the ethyl phosphotriester in the DNA template but were possibly due to an apparent adduct on the guanine bases.

E) *Mass spectrometry of the unmodified and phosphotriester DNA samples.*

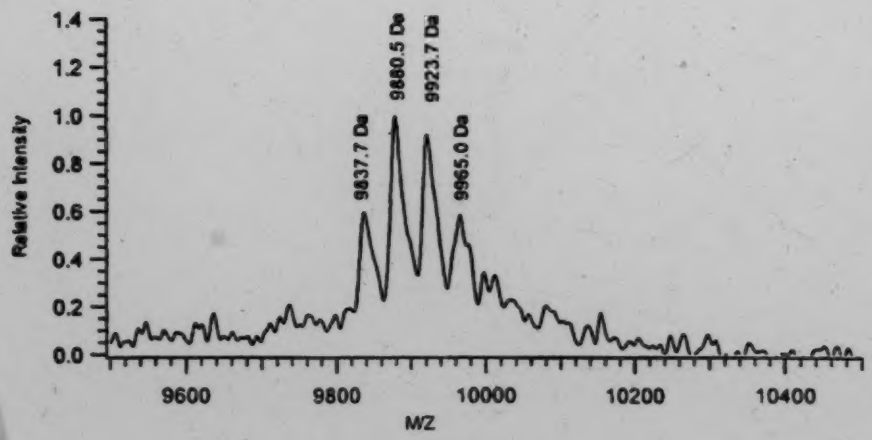
To confirm that additional adducts were present in the oligonucleotides, mass spectrometry was performed by Dr. Liang Li (Department of Chemistry, University of Alberta). Since mass spectrometry can differentiate oligonucleotides based on mass, any additional moiety on the DNA is observed as additional peaks, instead of a single peak for DNA containing a single ethyl phosphotriester. The peak corresponding to the oligonucleotide with an additional moiety exhibits a higher mass value. Thus, the difference between the mass value of the oligonucleotide containing the single ethyl phosphotriester and the mass value of the oligonucleotide containing the ethyl phosphotriester and a second unknown adduct, can be used to determine the identity of the unknown adduct. The DNA template containing a mixture of Rp and Sp ethyl phosphotriesters and the unmodified control template (C1), were deprotected in concentrated aqueous ammonia for 20 minutes at 65 °C and were analyzed to determine if the phosphotriester DNA contained an additional adduct. The unmodified control template (C1) analyzed here has the same sequence as PT2R, PT2S, and C2, but

Figure 13: Mass spectrometry of the unmodified control DNA and DNA containing a mix of Rp and Sp ethyl phosphotriesters. Panel A: Mass spectrum of the unmodified control DNA which has the same sequence as C2 but was not synthesized with the Pac protecting groups and therefore serves as a mass marker. Panel B: Mass spectrum of DNA containing a mix of Rp and Sp ethyl phosphotriesters, which was synthesized with the Pac protecting groups. The mass value that each peak represents is located above the corresponding peak.

A


9808.3 Da
Relative Intensity
1.0
0.8
0.6
0.4
0.2
0.0
9600
9800
10000
10200
10400
M/Z

B


9837.7 Da
9880.5 Da
9923.7 Da
9965.0 Da
Relative Intensity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
9600
9800
10000
10200
10400
M/Z

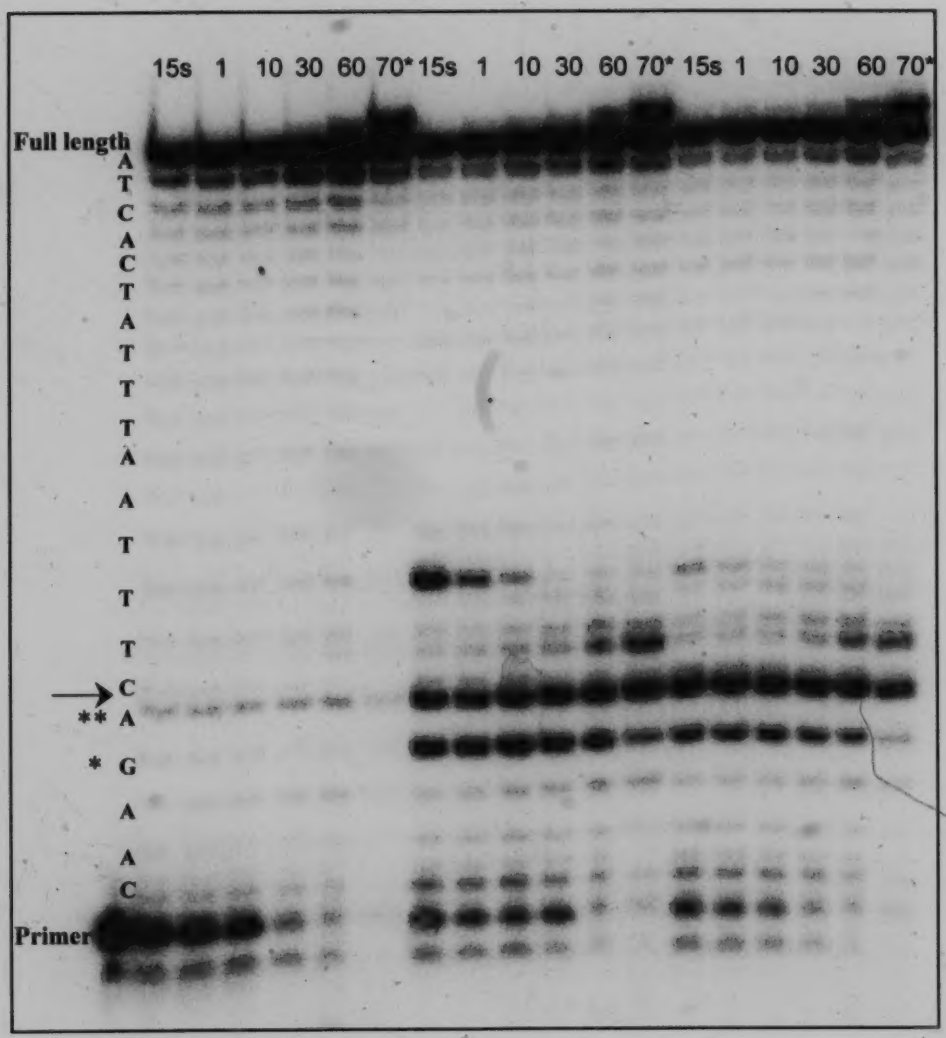
was not synthesized by the same procedure and, therefore, serves as a mass marker. This was done to determine the mass of the DNA templates, according to sequence composition, without the additional mass of any protective groups.

Figure 13 (panel A) is the mass spectrum obtained with the unmodified control template. The data clearly show a single mass value of 9808.3 Da, which represents the mass of the control template according to its sequence composition. In contrast, mass spectrum obtained for the phosphotriester DNA shows four peaks (Figure 13, panel B). The peak corresponding to a mass of 9837.7 Da represents an oligonucleotide containing a single ethyl phosphotriester. The three additional peaks appear to increase in mass in increments of 42.8 Da. Thus, up to three additional adducts with a mass of 42.8 Da each were present in our phosphotriester DNA sample and these adducts are likely acetyl groups on the guanine bases.

F) *Primer extension assay with D112A/E114A exonuclease deficient T4 DNA polymerase, using 3.5 hour deprotected C2, PT2R, and PT2S as templates.*

The acetyl groups present on the DNA substrate may have been a result of a substitution reaction occurring between acetic anhydride and the isopropylphenoxyacetyl protective groups on the guanine bases (Chaix *et al.*, 1989). Thus, we hypothesized that these acetyl groups might be removed by a harsher deprotection treatment. The oligonucleotides, C2, PT2R, and PT2S were deprotected with concentrated aqueous ammonia for 3.5 hours at 65°C. If deprotection removes all of the additional acetyl groups from template guanosines, then the bands corresponding to DNA polymerase pausing, located immediately 3'

Figure 14: Primer extension assay with D112A/E114A exonuclease deficient T4 DNA polymerase using 3.5 hour deprotected C2, PT2R, and PT2S as templates. Panel A: Exo⁻ DNA polymerase at 2 nM was incubated with the deprotected control template, C2 at 1 nM. Panel B: Exo⁻ DNA polymerase was incubated with the deprotected PT2R template. Panel C: Exo⁻ DNA polymerase was incubated with the deprotected PT2S template. The DNA templates, C2, PT2R, and PT2S were deprotected for 3.5 hours in concentrated aqueous ammonia at 65 °C. Reactions were run in the presence of all four dNTPs and aliquots of the reactions were stopped at the time points located above each lane. Time points are in minutes with the exception of the 15-second time point (15"). After 60-minutes of reaction time, 0.5 µl of 14 µM Exo⁻ DNA polymerase was added to the reaction and the reaction was stopped 10 minutes later (Lane 70*). The bands corresponding to blocked DNA replication two nucleotides and one nucleotide before the ethyl phosphotriester are indicated by * and ** respectively. The sequence of nucleotides added to extend the primer is provided on the left of the gel, along with the position of the ethyl phosphotriester, which is indicated by an arrow.


A
B
C
Unmodified
Rp
Sp
15s 1 10 30 60 70* 15s 1 10 30 60 70* 15s 1 10 30 60 70*
Full length
ATCACTATTTTAATTTTC
**A
*GAAC
Primer

to the template guanines should no longer be present using the unmodified template, C2. With the phosphotriester DNA templates, PT2R and PT2S, the –3 and –5 bands are expected to be absent since replication blocks at these positions are believed to be due to modified guanine bases in the template.

The primer, P2, was labeled at the 5' end with ^{32}P and annealed to the deprotected templates. Exo$^-$ T4 DNA polymerase, at 2 nM, was used to test the ability of these deprotected templates to direct DNA replication. At the 60-minute time point, 0.5 µl of 14 µM Exo$^-$ T4 DNA polymerase was added to the reaction mix and 10 minutes later the reactions were stopped. A high concentration of enzyme was used to facilitate the bypass of the ethyl phosphotriester and any remaining acetyl groups.

With the unmodified template, C2, Exo$^-$ DNA polymerase extended most of the primer to full length product in 30 minutes (Figure 14, panel A). None of the previously observed bands corresponding to blocked DNA replication were seen in this assay. Within 60 minutes most of the primer was extended to full length and full length + 1 nucleotide products. After the addition of a high concentration of Exo$^-$ all of the primer was extended to full length and full length + 1 nucleotide products (Figure 14, panel A, lane 70*).

Using the PT2R template, Exo$^-$ DNA polymerase produced some full length product but appeared to be paused or blocked at several locations (Figure 14, panel B). DNA replication by Exo$^-$ DNA polymerase was blocked at the nucleotide immediately before the Rp-ethyl phosphotriester (block indicated by **, the position of the ethyl phosphotriester is indicated by an arrow). The

intensity of the band corresponding to this block did not decrease with increasing reaction time, indicating that this substrate could not be extended by Exo⁻ DNA polymerase. Exo⁻ DNA polymerase also paused two nucleotides before the Rp-ethyl phosphotriester (indicated by *) and the intensity of this band slowly decreased with increasing reaction time, particularly after the addition of a high concentration of enzyme. Additionally, Exo⁻ DNA polymerase was no longer blocked at the –3 and –5 positions, implying that deprotection of the template guanines appeared to be successful at these positions. However, a novel band located three nucleotides after the phosphotriester was observed, and was absent after 30 minutes of reaction time (Figure 14, panel B, darker band three bands above position of ethyl phosphotriester). Moreover, after the addition of a high concentration of enzyme the proposed misincorporation band, located two nucleotides after the Rp-ethyl phosphotriester, appeared (Figure 14, panel B, lane 70*). This band was produced in small amounts by Exo⁻ DNA polymerase on the unmodified template, C2, but appeared to be enhanced by the presence of an Rp-ethyl phosphotriester. At the 70 minute time point, Exo⁻ DNA polymerase was able to produce mainly full length and full length + 1 nucleotide product. However, a large proportion of the DNA in the phosphotriester DNA sample, PT2R, blocked DNA replication by Exo⁻ at the position of the Rp-ethyl phosphotriester.

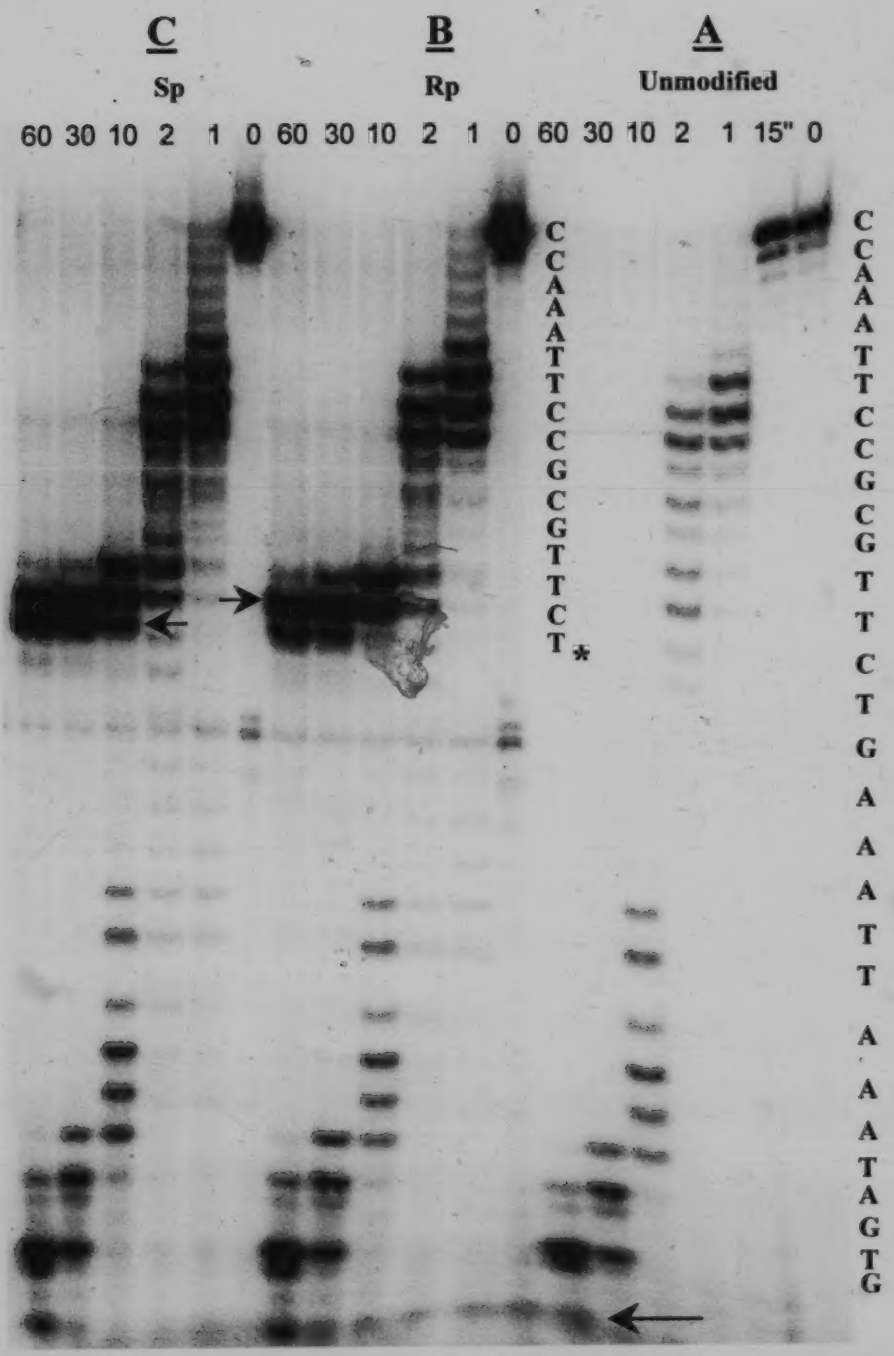
Similar results were obtained when the PT2S template was used (Figure 14, panel C). The only difference between DNA replication on PT2R and PT2S was that the novel pause band, located 3 nucleotides after the ethyl phosphotriester

was produced to a greater extent with PT2R (Figure 14, panel B, darker band 3 bands above position of ethyl phosphotriester). With PT2S, this pause band comprised a very small proportion of all extension products and its intensity was decreased to the background level by 1 minute (Figure 14, panel C, 1 minute lane).

G) *Single-stranded DNA exonuclease assay with wild type T4 DNA polymerase using 3.5 hour deprotected C2, PT2R, and PT2S as substrates.*

Previous experiments have indicated that phosphotriesters can also be hydrolysed under strong alkaline conditions (Kosolapoff, 1950; Shooter, 1976). Therefore, the oligonucleotides that had been deprotected for 3.5 hours in concentrated ammonia were tested with wild type T4 DNA polymerase to determine if the ethyl phosphotriester was still intact. In this experiment, the deprotected oligonucleotides, C2, PT2R, and PT2S were 5'-end labelled with ^{32}P. These labelled DNA substrates were incubated with 0.1 nM wild type T4 DNA polymerase. At various time points, aliquots of the reaction mix were stopped and the degradation products were separated by gel electrophoresis on a 15% polyacrylamide-7M urea gel. Wild type T4 DNA polymerase degraded the unmodified DNA, down to the dinucleotide within one hour (Figure 15, indicated by an arrow at the bottom of panel A). A low concentration of wild type T4 DNA polymerase was used to prevent complete degradation of the unmodified DNA and to produce a DNA size ladder to aid in the determination of the sequence of degradation products.

Figure 15: Single-stranded DNA exonuclease assay with wild type T4 DNA polymerase using 3.5 hour deprotected C2, PT2R, and PT2S as substrates. Panel A: Wild type T4 DNA polymerase (0.1 nM) was incubated with labelled C2 (1 nM). Panel B: Wild type T4 DNA polymerase was incubated with labelled PT2R. Panel C: Wild type T4 DNA polymerase was incubated with labelled PT2S. The DNA substrates C2, PT2R, and PT2S were deprotected for 3.5 hours in concentrated aqueous ammonia at 65 °C. All reactions were run in the absence of dNTPs and aliquots of the reactions were stopped at the time points located above each lane. Time points are in minutes with the exception of the 15-second time point (15"). The sequence of the unmodified DNA substrate is provided on the right of the gel, while the sequence of PT2R and PT2S is only provided up to the ethyl phosphotriester (indicated by *) since bands below this point are degraded unmodified DNA. Unmodified DNA degraded down to the dinucleotide is indicated by an arrow near the bottom of Panel A. An arrow located centrally in Panel B points to DNA degraded up to two nucleotides before the Rp-ethyl phosphotriester, while the arrow in Panel C indicates DNA degraded to one nucleotide before the Sp-ethyl phosphotriester.


C
Sp
B
Rp
A
Unmodified
60 30 10 2 1 0 60 30 10 2 1 0 60 30 10 2 1 15" 0
CCAAATTCCGCGTTCT*
CCAAATTCCGCGTTCTGAAATTAAATAGTG

Using PT2R as a substrate, wild type DNA polymerase was blocked at the nucleotide 3' to the ethyl phosphotriester (Figure 15, panel B, ethyl phosphotriester indicated by *). Bands due to 3'→5' exonuclease pausing were observed as far as three nucleotides before the ethyl phosphotriester. In particular, a large proportion of DNA was degraded down to two nucleotides before the ethyl phosphotriester (Figure 15, panel B, indicated by an arrow). This band comprised 65% of all degradation products at 60-minutes, as determined by densitometry. The band corresponding to DNA degraded up to the nucleotide immediately before the ethyl phosphotriester comprised 25% of all degradation products.

PT2S degradation by wild type DNA polymerase resulted in a majority of products degraded down to one nucleotide before the ethyl phosphotriester (Figure 15, panel C, indicated by an arrow). In fact, at 60 minutes this band represented 55% of all degradation products. DNA that had been degraded to two nucleotides before the ethyl phosphotriester comprised 35% of all degradation products. Thus, it appears as if wild type DNA polymerase had more difficulty degrading DNA near the Rp isomer than the Sp isomer. Bands below the position of the ethyl phosphotriester block represent residual unmodified DNA in the phosphotriester DNA samples and these bands run parallel with the bands corresponding to degraded unmodified DNA products of the same size. With both PT2R and PT2S samples, these smaller degradation products comprised only 10% of all degradation products. Therefore, the ethyl phosphotriester remained intact in 90% of the DNA after the 3.5-hour deprotection treatment.

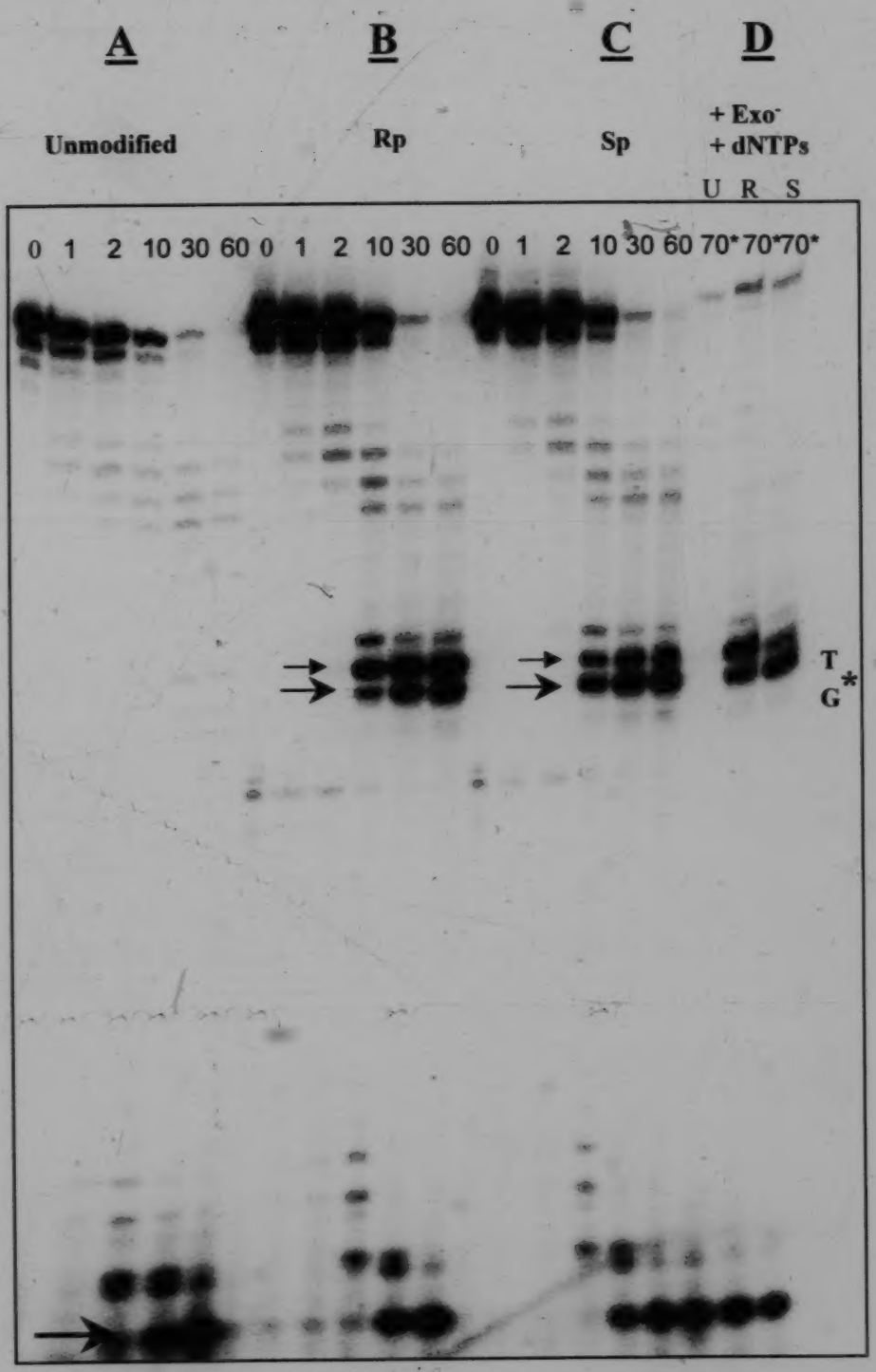
H) *Double-stranded DNA exonuclease assay with wild type T4 DNA polymerase using 3.5 hour deprotected C2, PT2R, and PT2S bound to complementary DNA as substrates.*

Because of our observations that wild type T4 DNA polymerase differed in approaching the Rp and Sp ethyl phosphotriester during single-stranded DNA degradation, degradation of double-stranded DNA containing the same templates (C2, PT2R, and PT2S) was also assayed. As opposed to single-stranded DNA, double-stranded DNA exists in a B-double helical structure where minor and major grooves are present (Watson and Crick, 1953). In this conformation the Sp isomer of the ethyl phosphotriester will protrude from the DNA backbone, in contrast to the Rp isomer, which lies in the major groove in closer proximity to the bases (Kan *et al.*, 1973; Weinfeld *et al.*, 1985).

In this assay, a complementary oligonucleotide (Comp1) containing a3'-phosphate was annealed to labelled C2, PT2R, or PT2S (Table 2). Wild type T4 DNA polymerase, at 1 nM, was added to 1 nM labelled duplex DNA. At various time points, aliquots of the reaction mix were removed and stopped. At the 60-minute time point, 0.5 µl of 14 µM Exo$^-$ T4 DNA polymerase and 100 µM dNTPs were added to the reaction mix to see if the degraded duplexes could be extended. Due to the 3'-phosphate synthesized on the complementary strand, wild type T4 DNA polymerase can only degrade the labelled template strand.

Wild type DNA polymerase degraded the unmodified duplex DNA substrate down to the dinucleotide in 60-minutes (Figure 16, panel A, indicated by an arrow). Double-stranded DNA degradation was much slower than that

Figure 16: Double-stranded DNA exonuclease assay with wild type T4 DNA polymerase using 3.5 hour deprotected C2, PT2R, and PT2S bound to complementary DNA as substrates. Panel A: Wild type T4 DNA polymerase (1 nM) was incubated with labelled deprotected C2 bound to complementary DNA (Comp1) (1 nM). Panel B: Wild type T4 DNA polymerase was incubated with labelled deprotected PT2R bound to Comp1. Panel C: Wild type T4 DNA polymerase was incubated with labelled deprotected PT2S bound to Comp1. Panel D: After 60 minutes of reaction time, 0.5 µl of 14 µM Exo$^-$ DNA polymerase and 100 µM dNTPs were added to the reaction and these reactions were stopped after 10-minutes (70*). Each lane represents the following: Lane U: Exo$^-$ and dNTPs added to degraded unmodified DNA, Lane R: Exo$^-$ and dNTPs added to degraded PT2R, Lane S: Exo$^-$ and dNTPs added to degraded PT2S. Reactions were initially run in the absence of dNTPs. Time points are provided above each lane and all time points are in minutes. The position of the ethyl phosphotriester in indicated by * and the two nucleotides surrounding the ethyl phosphotriester are provided. In panel A, unmodified DNA degraded down to the dinucleotide is indicated by an arrow. In panels B and C, a large arrow points to DNA degraded to one nucleotide before the ethyl phosphotriester and the smaller arrow indicates DNA degraded to two nucleotides before the ethyl phosphotriester.


A
B
C
D
Unmodified
Rp
Sp
+ Exo⁻
+ dNTPs
U R S
0 1 2 10 30 60 0 1 2 10 30 60 0 1 2 10 30 60 70* 70* 70*
T
G*

observed for single-stranded DNA degradation, as expected (Huang and Lehman, 1972). With unmodified single-stranded DNA, no full length DNA was left after one minute (Figure 15, panel A, top of gel). In contrast, with the double-stranded DNA substrate, some full length remained after 30-minutes of reaction time (Figure 16, panel A, top of gel).

With the phosphotriester DNA duplexes, wild type DNA polymerase was blocked primarily at the nucleotides immediately 3' to the ethyl phosphotriester (Figure 16, panels B and C, position of ethyl phosphotriester indicated by *). These results were similar to those obtained for single-stranded phosphotriester DNA degradation. Degradation of PT2R primarily occurred up to two nucleotides before the ethyl phosphotriester, whereas PT2S is degraded primarily up to one nucleotide before the ethyl phosphotriester (Figure 16, panels B and C, arrows).

After the addition of dNTPs and Exo$^-$ DNA polymerase at 60-minutes, the unmodified DNA substrate was not extended, as expected since most of the unmodified DNA was degraded down to the dinucleotide and could not provide the required primer terminus for DNA synthesis (Figure 16, panel D, lane U). Since PT2R and PT2S were degraded partially to two nucleotides and one nucleotide before the ethyl phosphotriester, leaving a 17–mer primer, they could have potentially acted as substrates for extension by Exo$^-$ DNA polymerase. In lanes R and S (Figure 16, panel D), we see that Exo$^-$ DNA polymerase was unable to extend both PT2R and PT2S back to full length. The amount of full length in these lanes was comparable to the amount of full length remaining in the

unmodified lane and was therefore, assumed to be background (Figure 16, panel D, lane U).

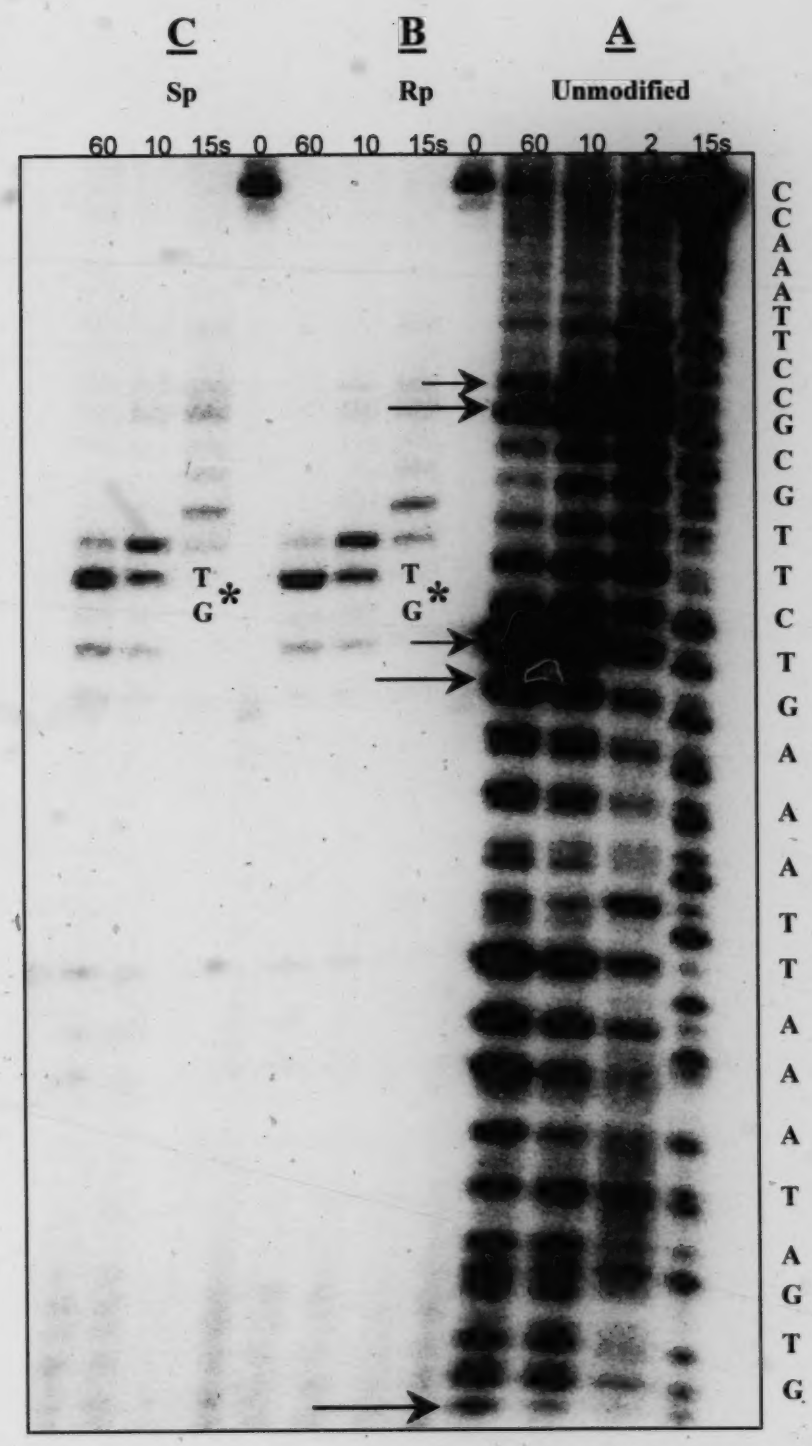
I) *Double-stranded DNA exonuclease assay with Escherichia coli Exonuclease III using 3.5 hour deprotected C2, PT2R, and PT2S bound to complementary DNA as substrates.*

Exonuclease III is the major apurinic/apyrimidinic endonuclease in *E. coli* (Levin *et al.*, 1988). This enzyme exhibits 3'→5' exonuclease, 3'-phosphomonoesterase, 3'-repair diesterase, and ribonuclease H activities specific toward double-stranded DNA or RNA/DNA duplexes (Henner *et al.*, 1983; Thomas and Olivera, 1978). Exonuclease III, however, dissociates frequently from DNA substrates during 3'→5' exonucleolytic degradation (Thomas and Olivera, 1978). Therefore, Exonuclease III was also tested to provide insight into the mechanism by which an ethyl phosphotriester inhibits 3'→5' exonuclease activity and slows the approaching exonuclease.

Reactions with complementary DNA (Comp1) annealed to labelled C2, PT2R, or PT2S (Table 2) were performed under similar reaction conditions to those used for the wild type T4 DNA polymerase double-stranded DNA exonuclease assay. Reaction products were separated by gel electrophoresis on a 15% polyacrylamide-7M urea gel.

Exonuclease III degraded unmodified duplex DNA down to the dinucleotide while producing many intermediate bands (Figure 17, panel A, dinucleotide indicated by an arrow). These intermediate bands were most likely

Figure 17: Double-stranded DNA exonuclease assay with *E. coli* DNA Exonuclease III using 3.5 hour deprotected C2, PT2R, and PT2S bound to complementary DNA as substrates. Panel A: Exonuclease III at 1 nM was incubated with unmodified DNA, C2, bound to complementary DNA (Comp1) at 1 nM. Panel B: Exonuclease III incubated with PT2R bound to Comp1. Panel C: Exonuclease III incubated with PT2S bound to Comp1. Reactions were run in the absence of dNTPs and stopped at the time points indicated at the top of each lane. Unmodified DNA degraded down to the dinucleotide is indicated by an arrow. In Panels B and C, large and small arrows point to DNA degraded to one nucleotide and two nucleotides before the ethyl phosphotriester, respectively. The sequence of the unmodified DNA template, C2, is provided on the right side of the figure in Panel A and the ethyl phosphotriester is indicated by * in Panels B and C.


C
Sp
B
Rp
A
Unmodified
60 10 15s 0 60 10 15s 0 60 10 2 15s
T
G*
T
G*
CCAAATTCCGCGTTCTGAAATTAAAATAGTG

due to the frequent dissociation of Exonuclease III from the DNA substrate. There were, however, four bands that were very prominent (indicated by large and small arrows) compared to the other intermediate bands. These bands correspond to exonuclease pausing at template guanines located in the middle of the template and ten nucleotides from the 3' end (see ladder). The band indicated by the larger arrow identifies the unmodified substrate degraded down to the guanine residue and the smaller arrow identifies the DNA substrate degraded down to one nucleotide before the guanine residue. Since Exonuclease III has 3'-phosphomonoesterase activity, the prominent bands corresponding to exonuclease pausing at the guanine located in the middle of the substrate may have been due to the simultaneous degradation of the complementary DNA (Comp1) and the labelled template. However, exonuclease pausing at the template guanine located ten nucleotides from the 3' end indicated that acetyl groups on the guanine residues might still have been present.

With the phosphotriester containing DNA substrates (PT2R and PT2S), Exonuclease III was unable to degrade PT2R and PT2S past the ethyl phosphotriester (Figure 17, panels B and C, ethyl phosphotriester indicated by *). In contrast to the degradation of phosphotriester DNA by wild type T4 DNA polymerase, no difference between the degradation of DNA containing the Rp isomer and the Sp isomer of an ethyl phosphotriester was observed with Exonuclease III. The amounts of DNA degraded up to two nucleotides and one nucleotide before the ethyl phosphotriester were equal for both PT2R and PT2S, as determined by densitometry.

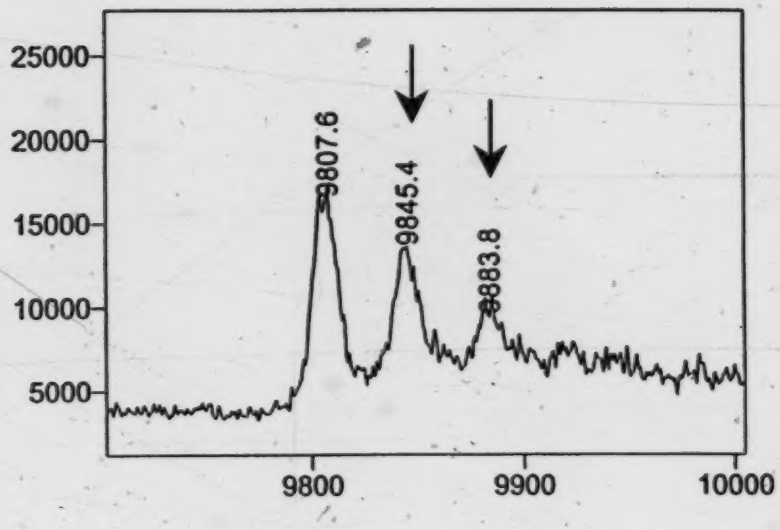
J) *Mass spectrometry of three hour deprotected C2 and phosphotriester DNA.*

Data obtained from the primer extension and exonuclease assays with the 3.5 hour deprotected DNA substrates, suggested the possibility of acetyl groups remaining on the guanine residues immediately 5' to the ethyl phosphotriester and ten nucleotides from the 3' end. An ethyl phosphotriester located between two thymines had no effect on DNA replication by Exo⁻ T4 DNA polymerase at 100 μM dNTPs (Figure 5, panel A). However, an ethyl phosphotriester located between a guanine and a thymine residue appeared to completely block DNA replication by Exo⁻ T4 DNA polymerase (Figure 14, panels B and C). Therefore, mass spectrometry on deprotected C2 and a phosphotriester DNA sample, containing a mix of Rp and Sp isomers, was done to determine if any additional acetyl groups remained in the DNA after a 3-hour ammonia treatment and was performed by Dr. Liang Li (Department of Chemistry, University of Alberta).

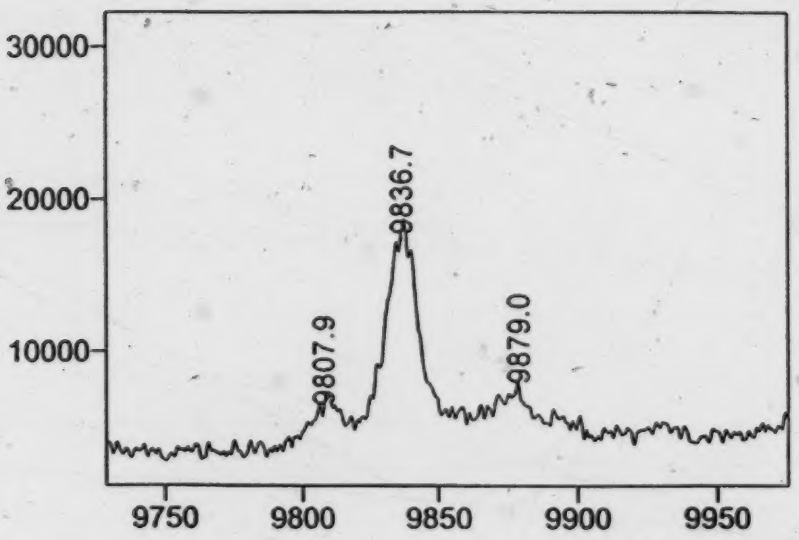
The data in Figure 18 (panel A) show the mass spectrum obtained for the 3-hour deprotected unmodified oligonucleotide, C2. A peak corresponding to an oligonucleotide with a mass value of 9807.6 Da was present, along with two potassium peaks. With the deprotected phosphotriester DNA, a major peak, corresponding to DNA containing a single ethyl phosphotriester, and two additional minor peaks were present (Figure 18, panel B). The minor peak, with a mass value of 9807.9 Da, corresponds to unmodified DNA in the phosphotriester DNA sample. Unmodified DNA was expected to be present since a 3-hour treatment with concentrated ammonia will cause some de-ethylation. The peak corresponding to an oligonucleotide with a mass of 9879.0 Da represented DNA

Figure 18: Mass spectrometry of 3-hour deprotected C2 and DNA containing a mix of Rp and Sp ethyl phosphotriesters. Panel A: The mass spectrum of deprotected C2. Panel B: The mass spectrum of deprotected DNA containing a mix of Rp and Sp ethyl phosphotriesters. The oligonucleotides were deprotected for three hours in concentrated aqueous ammonia at 65 °C prior to mass spectrometry. In Panel A, potassium peaks are indicated by arrows and all mass values are located above their corresponding peak.

A


25000
20000
15000
10000
5000
9807.6
9845.4
9883.8
9800
9900
10000

B


30000
20000
10000
9807.9
9836.7
9879.0
9750
9800
9850
9900
9950

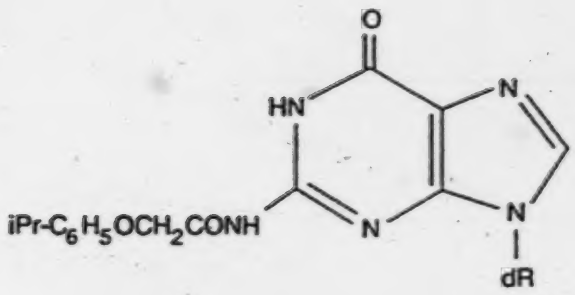
containing both a single ethyl phosphotriester and an acetyl group with a mass of 42.3 Da. The majority of the DNA in the sample analyzed here, however, contained a single ethyl phosphotriester represented by the 9836.7 Da peak.

Discussion: Part 2.

The phenoxyacetyl (Pac) (Figure 19) protecting groups were chosen because of their lability under the alkaline conditions used to release oligonucleotides from the solid phase after synthesis (Schulhof *et al.*, 1987), and thereby minimize alkali-catalyzed cleavage at the ethyl phosphotriester. Unfortunately, we were not made aware, by the suppliers of the Pac-protected nucleotides, of the side reaction of an exchange between phenoxyacetyl groups and acetyl groups, which occurs when acetic anhydride is used as the reagent to cap unreacted 5'-OH groups at the end of each cycle of nucleotide addition (Chaix *et al.*, 1989; Sinha *et al.*, 1993). (Ideally, phenoxyacetic anhydride should have been used for this purpose).

Our initial results with 20-minute deprotected DNA templates showed marked chain-termination at sites opposite template guanines; including the guanine immediately before the ethyl phosphotriester. Analysis by mass spectrometry revealed the presence of several species most of which contained one or more acetyl groups (Figure 13, panel B). In fact, less than 20% of the material was the fully deprotected ethyl phosphotriester oligonucleotide. However, our results indicated that acetylation of the N-2 exocyclic amino group of template guanine hindered replication by T4 DNA polymerase and may have promoted misincorporation. This is not entirely surprising since the N-2 position of guanine is a base-pairing position and is required for hydrogen bonding with cytosine.

Figure 19: Structure of isopropylphenoxyacetyl dG phosphoramidite.



iPr-PAC dG phosphoramidite

The removal of N^2-acetyl groups requires more prolonged hydrolysis than phenoxyacetyl groups. Preliminary experiments by Dr. M Weinfeld indicated that a 3-hour additional incubation in concentrated aqueous ammonia at 65 °C would be sufficient. However, the marked biphasic response observed with the DNA templates that were deprotected for 3.5 hours (Figure 14, panels B and C) make it unclear whether the observed DNA replication blocks and the possible misincorporation event were a result of an ethyl phosphotriester located between guanine and thymine residues in the DNA template or a combination of an ethyl phosphotriester and N^2-acetyl-2'-guanine in the DNA template.

A similar lesion, N^2-ethyl-2'-guanine, was shown to promote the misincorporation of guanine, resulting in G→C transversions (Matsuda *et al.*, 1999). An increase in this particular type of mutation was observed in human cells treated with acetaldehyde, which is produced through the metabolic oxidation of ethanol (Matsuda *et al.*, 1998). Acetaldehyde promotes the formation of N^2- ethyl-2'- guanine (Vaca *et al.*, 1995) and has been implicated in the development of several types of carcinomas (IARC, 1985).

Summary and Future Directions

Our results show that an ethyl phosphotriester in a DNA template slows DNA replication, possibly by impeding the formation of the Enzyme:DNA:dNTP complex required for nucleotide incorporation. This shifts the balance between polymerase and exonuclease activity towards exonuclease activity and prolongs "idling" by T4 DNA polymerases with a potent 3'→5' exonuclease activity, typical of replicative DNA polymerases. DNA polymerases that have a greater opportunity to proofread were observed to idle at the position of the ethyl phosphotriester, while those DNA polymerases with a reduced capacity to proofread bypassed the lesion with greater ease. *In vivo*, a stalled DNA polymerase at the site of an ethyl phosphotriester will likely dissociate and resume replication downstream resulting in the creation of daughter strand gaps. These gaps may be filled by bypass polymerases or completed via recombination. If these daughter strand gaps are left unrepaired, chromosome breakage will likely occur due to the exposure of single-stranded DNA.

In contrast to DNA replication by T4 DNA polymerase, an ethyl phosphotriester in the DNA template appeared to affect *E. coli* DNA polymerase I by a different mechanism. In this case, an ethyl phosphotriester two nucleotides from the primer terminus may have enhanced dissociation or inhibited binding of *E. coli* DNA polymerase I at the terminal template position.

To further investigate the mechanism by which an ethyl phosphotriester impedes the formation of the Enzyme:DNA:dNTP complex and slows elongation,

additional T4 DNA polymerase mutants could be tested. For example, mutant DNA polymerases defective in initiating the transfer of DNA from the polymerase active site to the exonuclease active site ("active site switching" mutants), such as the L412M-T4 DNA polymerase, would be good candidates. The T4 DNA polymerase mutant, L412M, exhibits increased binding of DNA in the polymerase active centre, resulting in decreased exonuclease activity and a mutator phenotype (Reha-Krantz and Nonay, 1994). The DNA spends more time in the polymerase active centre of L412M DNA polymerase than in the exonuclease active centre and, therefore, this mutant has a greater opportunity than wild type T4 DNA polymerase to form the ternary complex. This mutant DNA polymerase is predicted to be able to bypass the ethyl phosphotriester with greater ease than wild type or Exo$^-$ DNA polymerase and will provide valuable information on the role of ternary complex formation in the replication bypass of an ethyl phosphotriester.

In addition, different DNA templates containing an ethyl phosphotriester could be constructed so that problems associated with persistent protective groups are eliminated. The ethyl phosphotriester could also be synthesized in proximity to another lesion, such as O^6-methylguanine or an abasic site, since a variety of lesions are present in alkylated DNA (Singer, 1976). Using a modified form of this template, *in vivo*-like conditions can be assayed by the addition of accessory proteins, such as the clamp and single-stranded DNA binding proteins, and/or by assaying the ability of bypass polymerases to replicate DNA containing an ethyl phosphotriester. In addition, the repair of other lesions in proximity to an ethyl phosphotriester can be assessed.

Another experiment that could be performed would be to treat the PT2S DNA template with the *E. coli* Ada protein. The Ada protein will remove the Sp-ethyl group from the DNA backbone, leaving other adducts, such as acetyl groups, on the PT2S DNA template to be detected by a primer extension assay. The Ada protein can also be used to remove the Sp-ethyl group from the DNA template, PT1*, which contains a mix of Rp and Sp ethyl phosphotriesters located between two thymine residues. Thus, any differences between the replication of DNA containing an ethyl phosphotriester in the Rp or Sp isomer can be observed, which would aid in explaining the production of the full length and full length-1 products when *E. coli* DNA pol I replicated phosphotriester-containing DNA (Figure 6, panel B).

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